Postmortem Changes in High-Energy Phosphate Compounds and Chemical Indices for Assessing Freshness of Fish and Shellfish(Dissertation_全文)

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Contents

Abbreviations

Ad.....adenine
ADP.....adenosine 5'-diphosphate
AdR.....adenosine
AEC.....adenylate energy charge
AMP.....adenosine 5'-monophosphate
ATP.....adenosine 5'-triphosphate
HPLC....high performance liquid chromatography
Hx.....hypoxanthine
HxR.....inosine
IMP.....inosine 5'-monophosphate
NMR.....nuclear magnetic resonance
PAr.....arginine phosphate
PCr.....creatine phosphate
PDE.....phosphodiester
PME.....phosphomonoester
pHi.....intracellular pH
Pi.....inorganic phosphate
SP.....sugar phosphate
Xt.....xanthine

Introduction

It is well-known that the extractive components of fish and shellfish consist of many kinds of chemical compound. Some of those are important as nutrients and/or flavor components (Konosu and Yamaguchi, 1982) in seafoods. From a physiological point of view, some are important for osmotic regulation (Sakaguchi and Murata, 1988), anaerobic tolerance (Sato, 1988), and energy metabolism (Yamanaka, 1988) in fish and shellfish. ATP, one of nucleotide, is essential for organisms. Supplying the energy, ATP is degraded upon the action of organism and is maintained at a certain level when the re-synthesis occurs in the organism (Atkinson, 1968).

There is usually a time lag before the meats are consumed after catching; therefore it is very important to perform scientific researches on the postmortem changes of fish and shellfish. There have been many studies on the postmortem changes of ATP in fish, and the postmortem pathway of ATP degradation has been proved to proceed as follows: ATP → ADP → AMP → IMP → HxR → HX (Saito, 1961; Uchiyama and Ehira, 1970). HxR and/or Hx, which accumulate soon after death, are shown to provide a useful index of storage time and eating quality for many fish species (reviewed by Burt et al., 1969; Hiltz et al., 1969). Additionally, IMP, a metabolic intermediate of ATP degradation, has been revealed to have a essential role of "umami" taste of fish muscle (Murata and Sakaguchi,1989).

In fish, stress, such as starvation and fatigue, before death is known to alter pre-rigor and/or rigor-mortis periods and to

cause postmortem biochemical changes for many fish species (Amano et al., 1953; Tomlinson et al., 1961; Jones et al., 1965; Nakayama et al., 1992). Anesthetics are often used for handling fish in the field and laboratory and for the transportation of live fish as well (Sekizawa, 1982), since they prevent fish from straggling. The high-energy phosphates, PCr and/or ATP, are generally kept at higher levels in the anesthetized fish than those in the straggled fish. However, Van den Thillart et al. (1989) reported the decrease of pHi and PCr as well as the accumulation of Pi in the muscle of anesthetized carp compared with those in the unstressed carp in the in vivo 31P-NMR spectra. Their results indicate that the anesthetic itself acts as one of stressor on fish. In the muscle of chicken, Khan (1974) reported that the treatment with anesthetics altered the postmortem biochemical changes. In fish muscle, there is little information available about the effects of anesthetics on the postmortem biochemical changes especially of ATP and other high-energy phosphate compounds.

One of the most important qualities of raw fish is freshness. The degree of freshness is expressed in terms of the K value which is obtained from concentrations of ATP and its related compounds (Saito et al., 1959). Although the K value is an effective indicator for the freshness of fish during storage, troublesome procedures including homogenization of the tissue, extraction and chromatographic measurement of the levels of compounds are required for measuring the K value. It seems to be an inaccurate indicator for fish kept within shorter periods, since the rates of changes in the K values were low (Jones et al., 1964; Uchiyama and Ehira, 1970).

^{31}P NMR is known as a non-invasive and convenient method for rapid determination of high energy phosphate compounds in living body (Gadian, 1982) and in various organs (Cohen, 1987). The NMR also facilitates the examination of functions in isolated organs. Probably, this method enables to evaluate fish meat quality, such as freshness, because of its non-invasive, convenient, and rapid determination of high energy phosphate compounds in the tissue of fish.

In the muscle of mollusk, the pathway of ATP degradation was reported to proceed as follows: ATP → ADP → AMP → AdR → HxR → Hx (Saito, 1961; Arai, 1961a and b), while the details on postmortem changes of ATP and its related compounds, such as the differences of pathways and degradation patterns among species and tissues and the effects of storage temperature on the postmortem changes of those compounds, have not been clear. Furthermore, the chemical assessment of freshness during postmortem storage has not been established for mollusk, although a large amount of mollusk are consumed as fresh materials for a variety of dishes, including such popular dishes as sashimi and sushi in Japan.

In the present studies, the author examined the effects of anesthetic stress on the postmortem changes of high-energy phosphates and ATP-related compounds in fish muscle, and the possibilities of non-invasive assessment of fish meat quality using ^{31}P NMR. The author also investigated the postmortem changes of high-energy phosphates and ATP-related compounds and discussed potential freshness index of mollusk, i.e., pelecypod, gastropod, and cephalopod.

Chapter I. Postmortem changes of high-energy phosphate compounds in carp muscle

Anesthetics are often used for handling fish in the field and laboratory and also used for the transportation of live fish (Sekizawa, 1982). However, most anesthetics are expensive and are not always safe for human and fish. High concentration of CO_2 is known to have an anesthetic effect on many animals including fish (Mayer et al., 1961; Klemm, 1964; Klemm, 1968; Mitsuda et al., 1982; Itazawa and Takeda, 1982). CO_2 seems to be a useful anesthetic for such a purpose as live fish transportation because of its safety for human consumption, effectiveness, and low cost, while the anesthetic level of CO_2 is well-known to reduce the oxygen affinity and oxygen capacity of the blood, and also decrease the blood of fish (Itazawa and Takeda, 1982; Mitsuda et al., 1982) and tissue pH of mammals (Meyer et al., 1961; Klemm, 1964; Klemm, 1968; Schindler and Betz, 1976). The lowering of the tissue pH is anticipated to reduce and/or disturb the tissue activity. In fact, the reduction of brain activity owing to a lowering of the brain pH has been reported in mammals by several workers (Meyer et al., 1961; Klemm, 1964; Klemm, 1968; Schindler and Betz, 1976). The reduction of metabolism by the decrease in intracellular pH has also been reported in mammals (Mallan, 1988; Geiser, 1988), lungfish (Delaney et al., 1977), land snail (Barnhart, 1986), sea-urchin eggs (Winkler, 1982), and brine shrimp embryos (Busa et al., 1982; Busa and Crowe, 1983). Moreover, Tappon (1971) observed that the muscle Pcr levels were

virtually unaffected under the condition of 15% CO_2 exposure (Tappon, 1971). Jackey and Shafer also reported that the hypercapnia (15% CO_2, 21%O_2, balance N_2) was characterized by the conservation of high-energy phosphates (Jackey and Shafer, 1971). On the other hand, Itazawa and Takeda reported the increase of oxygen debt in the carp anesthetized with mixed bubbling of CO_2 and O_2 gas (Itazawa and Takeda, 1982).

The author previously demonstrated the efficacy of cold-CO_2 anesthesia in long-term anesthesia of adult carp supposing the transportation of live fish (Yokoyama et al., 1989). The carp anesthetized with cold-CO_2 was kept in a favorable anesthetic state safely for 10 h. However, the metabolic state of the anesthetized carp is unclear. It is interesting to determine whether the anesthesia changes, reduces, and/or disturbs the energy state in the carp or not.

Stress including starvation and fatigue before death is known to change pre-rigor and/or rigor-mortis periods, and to cause postmortem biochemical changes for many fish species (Amano et al., 1953; Tomlinson et al., 1961; Jones et al., 1965; Nakayama et al., 1992). Similar findings have also been reported in the muscle of chicken treated with anesthetics (Khan, 1974). However, there is little information about the effects of anesthetics on the postmortem biochemical changes such as levels of high-energy phosphate compounds or ATP and its related compounds in fish muscles. After the live fish is killed, there is usually a time lag before the fish meat is consumed. Thus, it is important to determine the postmortem changes of the meat quality of the carp anesthetized.

One of the most important quality of fish is freshness. Degree of freshness is expressed in terms of the K value which is obtained from concentrations of ATP and its related compounds (Saito et al., 1959). The K value is calculated from the percentage of HxR plus Hx to breakdown products from ATP to Hx. Jones et al., (1964) improved the index of fish freshness by measuring Hx concentrations from the fish body. Although estimation of the degree of freshness by Hx concentration is an effective indicator for fish during storage, it is troublesomely necessary for the measurement to homogenize the tissue, extract and measure the levels of compounds by chromatography. And Hx level is not an accurate indicator for fish kept within short periods (Jones et al., 1964; Uchiyama and Ehira, 1970).

Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) is a non-invasive method for rapid determination of energy levels in living body (Gadian, 1982). This technique is convenient for examining the metabolism of high energy phosphate compounds and has been used for that purpose in various organs (Cohen, 1987). It is also facilitate to examine the functions of isolated organs.

In this chapter, first the author states the postmortem changes in the contents of ATP and its related compounds examined in the muscle of carp of five treatment groups, cold-CO_2, 14°C-O_2, 23°C-O_2, CO_2-recovery, and control groups, using HPLC. The differences in physiological state between anesthetized and unanesthetized carp were discussed. The postmortem changes in freshness of carp muscle were also discussed in relation to meat quality by means of HPLC. Using ^{31}P NMR, the author states secondly the evaluation of anesthetic effects on carp muscle and of degree of fish freshness

in relation to high-energy phosphates. The postmortem pH and glycogen levels were also measured in the muscle of cold-CO_2 and control groups. The author also evaluates the meat quality by ^{31}P NMR.

I.1 Postmortem changes of ATP and its related compounds measured by HPLC

Anesthetics are often used for handling fish in the field and laboratory and for the transportation of live fish as well (Sekizawa, 1982). It has been reported that the treatment with anesthetics produces the mode of postmortem biochemical changes in the muscle of chicken (Khan, 1974). In fish, stress, such as starvation and fatigue, before death is known to alter pre-rigor and/or rigor-mortis periods, and to cause postmortem biochemical changes for many fish species (Amano et al., 1953; Tomlinson et al., 1961; Jones et al., 1965; Nakayama et al., 1992). There is little information, however, about the effects of anesthetics on the postmortem biochemical changes especially of ATP and other high-energy phosphate compounds in fish muscles.

In this section, the author first describes the pathway of ATP breakdown in the carp muscle, ATP → ADP → AMP → IMP → HxR → Hx, as reviewed by Saito (1961) and Uchiyama and Ehira (1970) in several kinds of fish. Secondly, the author investigated the postmortem changes in the contents of ATP and its related compounds in the muscle of carp of five treatment groups, cold-CO_2, 14°C-O_2, 23°C-O_2, CO_2-recovery, and control groups, by means of HPLC. The differences in physiological state between anesthetized and unanesthetized carp were discussed. The postmortem changes of ATP and its related compounds and in freshness of carp muscle were also discussed in relation to meat quality by means of HPLC.

Materials and Methods

The experiments were carried out on 50 carp Cyprinus carpio, weighing about 500g (487±39g, mean±S.D.) each. Each group of 10 carp was reared in a 1000 l tank placed indoors, and acclimated to 23±1°C under a 14 h-light and 10 h-dark cycle for at least two months. They were daily fed a commercial diet, but were fasted one day prior to the experiment. In the case of cold-CO_2 treatment, ten carp were anesthetized at 4°C for 30min and then placed in an experimental container (25 x 50 x 50 cm, 60 l) maintained in advance at 14°C and Pco_2=80mmHg by bubbling 11% CO_2 and 89% O_2 gas at 2.0 l/min for the following 9.5 h Yokoyama et al., 1989a and 1989b). Immediately after the total of 10-h of the cold-CO_2 anesthesia, they were decapitated (cold-CO_2 group). In the case of 23°C-O_2 treatment, ten carp were anesthetized at 4°C for 30min, and then placed in the container at 23°C for the following 9.5 h, bubbling pure O_2 gas (2.0 l/min). Immediately after the treatment, they were decapitated (23°C-O_2 group). In the case of 14°C-O_2 treatment, ten carp were anesthetized at 4°C for 30min and then placed in the container at 14°C for the following 9.5 h, bubbling pure O_2 gas (2.0 l/min). Immediately after the treatment, they were decapitated (14°C-O_2 group). In the case of CO_2-recovery treatment, ten carp were cold-CO_2 anesthetized for 10 h as described above and then returned to the rearing tank kept at 23°C for recovery. On the next day after the 10-h of cold-CO_2 treatment, they were gently netted and decapitated immediately (CO_2-recovery group). As a control, ten carp were gently netted from the rearing tank and decapitated immediately (control group). White muscle was

dissected from the dorsal part of the carp and held in a glass bottle with ground stopper (o10 x 10cm). The storage temperature was kept at 23°C. At each sampling, approximately 0.5 g of muscle from the anterior end of the muscle was cut and discarded. Subsequently, from the remaining muscle piece, a 1 g-portion of muscle was newly cut and was submitted for extraction and determination of ATP and its related compounds and glycogen.

Extraction and Determination of ATP and Its Related Compounds

The extraction and the determination of ATP and its related compounds by HPLC was carried out in the same manner as reported by Ryder (Ryder, 1985).

Calculation of K Value and Adenylate Energy Charge

The K value as a freshness index (Saito et al., 1959) and the adenylate energy charge (AEC) proposed as a metabolic regulatory parameters (Atkinson, 1968) were calculated from the contents of ATP and its related compounds from the following equations:

$$K\ (\%) = (HxR + Hx) / (ATP + ADP + AMP + IMP + HxR + Hx) \times 100$$

$$AEC = 1/2\ (2ATP + ADP) / (ATP + ADP + AMP)$$

Results and Discussion

Postmortem Changes of ATP and Its Related Compounds in Carp Muscle

Figure I-1 shows the changes of ATP and its related compounds in the carp muscle during storage at 23° C. ATP level decreased during storage. The levels of ADP and AMP were low, while IMP accumulated during storage. The rates of HxR and Hx accumulation were slow compared with that of IMP. From these results, the author

confirmed the pathway of ATP-degradation, ATP → ADP → AMP → IMP → HxR → Hx, and the limiting step of ATP-breakdown, IMP → HxR, in the muscle of carp (reviewed by Saito, 1961, and Uchiyama and Ehira, 1970).

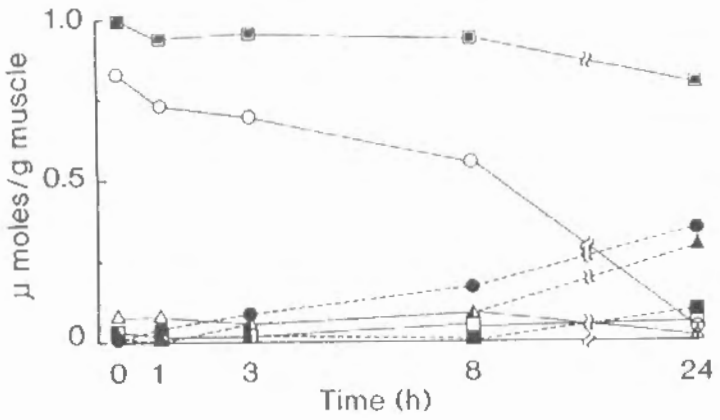

Fig. I-1 Changes in average content of ATP and its related compounds in carp muscle (n=10) during storage at 23°C.
○, ATP; △, ADP; □, AMP; ●, IMP; ▲, HxR; ■, Hx; ▣, Total.

Effects of Stress (Cold-CO_2 Anesthesia) on Physiological State of Carp Muscle at the Time of Death

Table I-1 shows the contents of ATP and IMP and energy charge values in the muscle of each five experimental group at the time of death. The ATP content of control group, about 7.3μmol/g, indicates that the carp was killed immediately. In the control group, the IMP content was low and the energy charge value was high. In the cold-CO_2 group, the ATP content was very low and the IMP content was very high compared with those in the control group. There were statistically significant differences in these values between the two groups ($p<0.01$, paired t-test). The energy charge value was also slightly, but significantly lowered by anesthesia ($p<0.05$). The changes in those values in the cold-CO_2

Table I-1. Initial values of ATP and IMP contents (μmol/g) and energy charge in each group.

Group	ATP	IMP	Energy charge
Control	7.3±1.0	0.3±0.2	0.93±0.03
Cold-CO_2	4.5±1.5*	1.8±1.0*	0.88±0.04*
23°C-O_2	6.6±0.9	0.2±0.3	0.94±0.03
14°C-O_2	7.0±0.6	0.1±0.1	0.94±0.02
CO_2-recovery	6.7±1.0	0.2±0.2	0.94±0.07

* Stastically significant at 5% level or better compared with control group. Values are expressed as mean±SD, n=10.

group seemed to be caused mainly by the anesthetic effect of CO_2, but not by cold temperature at 14°C, since the 14°C-O_2 group showed little difference in those values from the control group (**Table I-1**). There were also little difference detected in ATP and IMP contents, and in energy charge values between the control and 23°C-O_2 groups. An increase in erythrocyte ATP level was reported by Timms and Mengel (1968) in rats exposed to hyperoxic conditions. However, the muscle ATP levels in the two O_2 groups were at the control level. The exact reason of this disagreement is not clear, but the differences of species and/or tissues are factors to be considered.

The decrease in the energy charge and ATP levels in the cold-CO_2 group at the time of death indicated that the anesthetic level of CO_2 changed the energy metabolism in the carp muscle. On the other hand, Jacey and Schaefer (1972) reported that the arterial ATP, ADP, and AMP contents, and the energy charge value were virtually unchanged in guinea pigs exposed to 15% CO_2 in 21% O_2 and balanced N_2. Similar findings were made by Granholm and Siesjo (1969) in the brain of cats acutely exposed to various concentrations of CO_2 up

to the level which produced arterial Pco_2 values of 100mmHg. They observed no changes in tissue ATP and ADP levels or in PCr content. However, these findings were on the mammals and the dose of CO_2 was below the anesthetic level. The decrease in ATP content and energy charge value, and the increase in IMP content in the cold-CO_2 group may partially be explained by the findings of Itazawa and Takeda (1982); there was an increase of oxygen debt in carp during CO_2-anesthesia. The anesthetic level of CO_2 is well known to reduce the oxygen affinity and oxygen capacity of the blood. The carp in the cold-CO_2 group seemed to be in a substantial hypoxic condition despite the high level of partial pressure of O_2 in water (Po_2=650mmHg) (Yokoyama et at., 1989a and 1989b). However, when the carp recovered from cold-CO_2 anesthesia, their energy state returned to the control levels (**Table I-1**).

Effects of Stress (Cold-CO_2 Anesthesia) on Postmortem Changes of Carp Muscle

Figure I-2a shows the postmortem changes in content of ATP and its related compounds in the control group. The ATP content gradually decreased during storage; being 0.4 and 1.2μmol/g lower in 1 h and 3 h postmortem, respectively, than the amount occurring initially. The contents of IMP and HxR was directly related to the extent of ATP breakdown. IMP and HxR thus showed a gradual increase during storage. ADP, AMP, and Hx were at low levels of less than 0.8μmol/g.

The changes in content of ATP and its related compounds in the cold-CO_2 group were markedly different from those in the control group as shown in **Fig.I-2b**. The ATP content in 1 h and 3 h

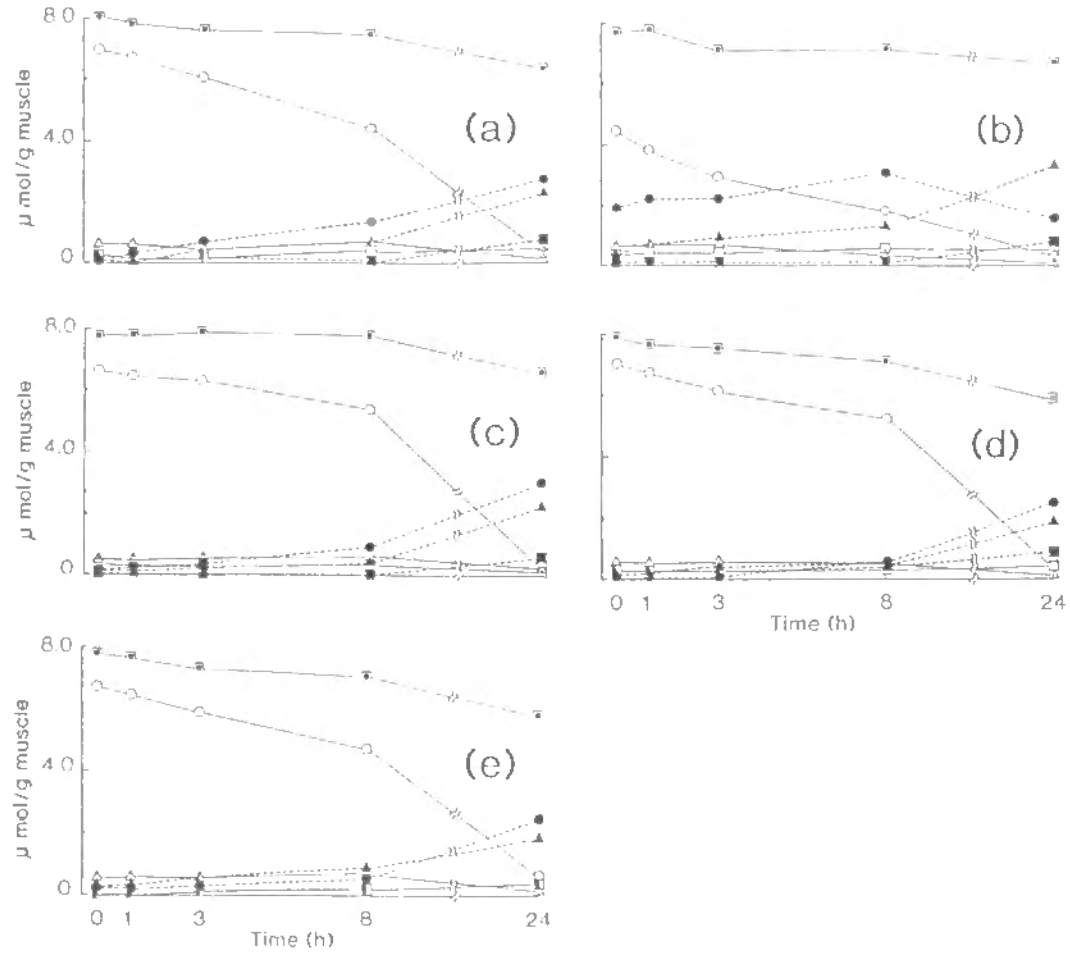

Fig. I-2 Changes in average content of ATP and its related compounds in carp muscle (n=10) during storage at 23°C. a, control group; b, cold-CO_2 group; c, 23°C-O_2 group; d, 14°C-O_2 group; e, CO_2-recovery group. Symbols are the same as those given in the footnote of Fig. I-1.

postmortem 0.7 and 1.5μmol/g lower, respectively, than that at 0 h. There were significant differences ($p<0.05$) in the decreasing rate of ATP contents observed during 1 h and 3 h postmortem between the control and cold-CO_2 groups. Nakayama et al. (1992) reported that the muscle of the carp which struggles severely prior to death is characterized by a lower ATP level at the time of death and followed by a more rapid decrease in the ATP level compared with those of unstressed carp. Their findings coincide with our

findings on ATP level and its postmortem breakdown. IMP gradually increased from 1.8 to 3.2 μmol/g during storage, and then it decreased. HxR increased gradually during storage, although the corresponding content at each storage time was higher in the cold-CO_2 group than that in the control group.

The changes in the contents of ATP-related compounds in the 23°C-O_2 and 14°C-O_2 groups were nearly the same as those observed in the control group (**Figs. I-2c and I-2d**). The ATP content was high and the breakdown proceeded gradually. IMP and HxR increased gradually just as observed in the control group (**Fig. I-2a**). These findings indicate that the carp in the container at 23°C or 14°C under bubbling pure O_2 gas were kept in an unstressed state. The contents and changes of ATP and its related compounds in the CO_2-recovery group were the same as those observed in the control group (**Fig.I-2c**). These findings on ATP and its related compounds indicate that the carp in the CO_2-recovery group recovered their energy state and muscle metabolism to the levels of control group. There would be little difference in the onset of rigor-mortis and its development between the control and the CO_2-recovery groups, since there was little differences in the levels and the changes of the ATP content and between the two groups (**Figs. I-2a and I-2c**), as reported by Iwamoto (1991).

Figure I-3 shows the changes in K value of carp muscle during storage. The specimen of all five groups kept for 8 h emitted a faintly putrid smell; this stage was recognized the stage of initial decomposition (Matsumoto and Yamanaka, 1990). The specimen kept for 24 h emitted an unpleasant odor and was recognized as the stage of advanced decomposition. The K value of cold-CO_2 group at

0, 1, 3, and 8 h was 5, 8, 14, and 19 %, respectively. The value of control group at each time was 3, 5, 7, and 13 %, respectively. Although the differences of the K values between the two group were not large, the estimation of the degree of freshness by the K value seemed to be accomplished since there were statistically significant differences ($p<0.01$) in the corresponding values between the control and cold-CO_2 groups. These results indicate that the anesthetic (cold-CO_2 anesthesia) affects the postmortem changes of fish freshness. The levels and changes in the K value in the 14°C-O_2, 23°C-O_2, and CO_2-recovery groups showed little difference from the control group. As a result, the change in

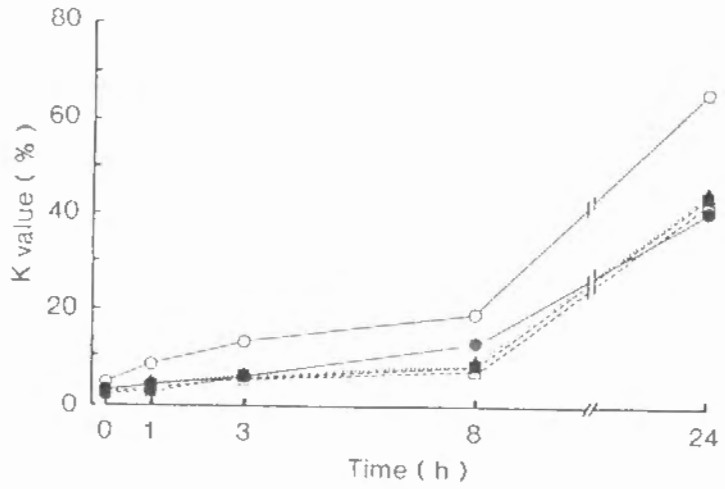

Fig. I-3 Changes in average K value of carp muscle (n=10) during storage.
▲, control group; ○, cold-CO_2 group; □, 23°C-O_2 group;
■, 14°C-O_2 group; ●, CO_2-recovery group.

freshness was the same in the carp that had recovered from anesthesia as in the control group. Additionally, the levels and changes in the ATP and its related compounds in the CO_2-recovery group indicate that the onset of rigor mortis and its development

in the carp recovering from anesthesia would be the same as those in the control group. Therefore, the meat quality of the carp anesthetized with cold-CO_2 anesthesia would be unaffected, if the carp is allowed to recover from the anesthesia before death.

I.2 Phosphorus-31 NMR study of post■orte■ energy ■etabolis■

In vivo phosphorus-31 nuclear magnetic resonance (^{31}P NMR) has been used so far for measuring serial changes in high energy phosphate metabolism (Gadian, 1982). In the muscle, not only concentrations of high energy phosphate metabolites but also intracellular pH and Mg^{2+} ion concentrations can be measured, which allows to evaluate the fatigue status of the muscle (Gupta and Gupta, 1987). Recently, the energy stores and intracellular pH in carp during anoxia were studied using ^{31}P NMR (Thillart, 1989). Changes in high energy phosphate metabolism of fish muscle was dependent on the dissolved oxygen concentration. In the last section (I.1), the author showed that the carp anesthetized with cold-CO_2 seemed to be under the substantially hypoxic conditions. To evaluate the conditions of anesthetized carp, it will be meaning to determine the high energy phosphates and pH in the carp muscle using ^{31}P NMR.

One of the most important qualities of raw fish is freshness. The degree of freshness is expressed in terms of the K value which is obtained from concentrations of ATP and its related compounds (Saito et al., 1959). Although the K value is an effective indicator for the freshness of carp during storage, it is troublesome for the measurement to homogenize the tissue, extract and separate the compounds each other by chromatography. And it seems not to be an accurate indicator for carp kept within short periods, since the rates of changes in the K values were slow as shown in **Fig. I-3**. Similar results was also reported by Jones et

al., (1964) and by Uchiyama and Ehira (1970).

^{31}P NMR is a non-invasive and convenient method for rapid determination of high energy phosphate compounds in living body (Gadian, 1982) and in various organs (Cohen, 1987). It is also facilitate examination of functions in isolated organs. If possible, ^{31}P NMR seems to be a possible method for evaluating fish meat quality, such as freshness, because of its non-invasive, convenient, and rapid determination of high energy phosphate compounds in the tissue of fish.

In this section, the author examined the high energy phosphate compounds, pH, and Pi in the carp muscle of cold-CO_2 and control groups using ^{31}P NMR and also glycogen contents by conventional chemical methods, and evaluated the effects of stress (cold-CO_2 anesthesia) on the energy metabolism of the carp. The author also discussed the efficacy of ^{31}P NMR to estimate the freshness of carp muscle.

Materials and Methods

Materials and Procedure of Anesthesia

The experiments were carried out on 10 carp Cyprinus carpio, weighing about 500g (498±23g, mean±S.D.) each. 10 carp were reared in a 1000 l tank placed indoors, and acclimated to 23±1°C under a 14 h-light and 10 h-dark cycle for at least two months. They were daily fed a commercial diet, but fasted one day prior to the experiment. In the case of cold-CO_2 treatment, five carp were anesthetized at 4°C for 30min and then placed in an experimental container maintained in advance at 14°C and Pco_2=80mmHg following

9.5 h in the closed system as described in Section I-1. Immediately after the total of 10-h of the cold-CO_2 anesthesia, they were decapitated (cold-CO_2 group). As a control, five carp were gently netted from the rearing tank and decapitated immediately (control group). White muscle block, the size of which was 3 cm by 3 by 1.5 thick, was dissected from the dorsal part of the carp and used for the NMR measurement, Immediately. The remaining muscle piece was stored at 23° C , at each fixed time of storage 0.5-g portion of muscle was newly cut and used for the determination of glycogen.

^{31}P NMR Measurement of Carp Muscle

NMR measurements were made with a JNM GSX-270 spectrometer system (JEOL, Japan). The surface coil (11 mm in diameter, five turn) for NMR detection was placed against the center of muscle block. The spectra were obtained at an operating frequency of 109.14 MHz for phosphorus. Radiofrequency pulses of 20 μsec duration were delivered every 1.5 sec for NMR acquisition. It took 5 min to accumulate 200 scans for each measurement. The muscle block was fixed on the NMR probe for 24 h, and spectra were obtained at 0, 1, 2, 3, 5, 8, 12, 16, 20, and 24 h. The criterion for chemical shift used was 0 ppm creatine phosphate. Changes in the concentration of each compounds were determined from the area of resonance line of each signal. Relative index of concentration of [Pi], [PCr], and [ATP (β-ATP)] was obtained by the ratios of [PCr]/[Pi] and [ATP (β-ATP)]/[Pi].

Determination of Intracellular pH

The intracellular pH (pHi) in fish muscle was estimated on the basis of the differences between the chemical shifts of Pi and of PCr by constructing a pH titration curve with a standard solution (H_3PO_4, 50 mM; PCr, 25 mM; KCl, 100 mM; and $MgCl_2$, 1 mM). The chemical shift of PCr was postulated as 0 ppm. pHi was calculated from the chemical shift (δppm) of the Pi resonance by use of the following equation (Gadian et al., 1979; Seo et al., 1983).

$$pHI = pK' + \log (\delta - \delta_\alpha) / (\delta_\beta - \delta)$$

in which pK' = 6.87, δ_α = -3.17 ppm, δ_β = -5.61 ppm were used.

Determination of Glycogen

After 0.5 g of muscle was extracted in 5 ml of heated 30% potassium hydroxide, 5 ml of absolute ethanol was added to the solution and glycogen was precipitated. The obtained glycogen was hydrolyzed with 1 M sulfuric acid and glucose formed was determined by the combination of mutarotase and glucose oxidase (Glucose C-Test, Wako).

Results and Discussion

Influence of Anesthetic Stress on NMR Spectrum

Typical ^{31}P NMR spectra of carp muscle of two groups obtained immediately after the decapitation are shown in **Fig. I-4.** There were five or six main peaks, assigned, from left to right, to SP + IMP (not detected in the muscle of control group), Pi, PCr, γ-phosphate of ATP + β-phosphate of ADP, α-phosphate of ATP + α-phosphate of ADP + NAD(H), and β-phosphate of ATP. These peak

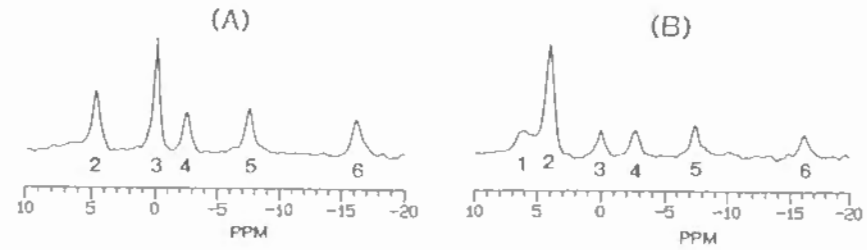

Fig. I-4 Typical ^{31}P NMR spectra of the carp muscle of control (A) and cold-CO_2 (B) groups immediately after death. Peak assignments are as follows: 1, SP + IMP; 2, PI; 3, PCr; 4, γ-phosphate of ATP + β-phosphate of ADP; 5, α-phosphate of ATP + α-phosphate of ADP + NAD(H), and 6, β-phosphate of ATP.

assignments were performed according to Barany and Glonek (1982). In the section I-1, the ATP content of control group was 7.3 μmol/g and the ATP level indicated that the carp was killed immediately (**Table I-1**), while the NMR spectra of control group showed the accumulation of PI. Van Den Thillart et al. (1989) reported that PI peaks of in vivo ^{31}P NMR spectra of the carp which is completely unstressed is normally barely visible. These results indicate that the netting, decapitation, and the timelag for setting the muscle to the NMR prove causes increased energy demand. However, there were much differences in the spectrum between the two groups. In the cold-CO_2 group, PCr and ATP levels decreased and the Pi level increased compared with those in control groups. Evaluation of the influence of anesthetic stress seems to be possible by the NMR measurement of dissected muscle.

Effects of Anesthetic Stress on the Physiological State of Carp Muscle at the Time of Death

Table I-2 shows the values of [PCr]/[Pi], [ATP]/[PI], pHi, and glycogen contents in the muscle of each two experimental group at the time of death. The [Pcr]/[PI] and [ATP]/[PI] in the cold-CO_2

group were significantly lower than those in control group ($p<0.05$). The anesthetic stress seemed to increase the energy demands of carp muscle. Itazawa and Takeda (1982) reported that there was an increase of oxygen debt in carp during CO_2-anesthesia. Van den Thillart et al. (1989) reported that anoxia caused a rapid decrease in PCr in carp, and that after the PCr store had been exhausted by more than 85%, the ATP level fell, whereas IMP accumulated markedly. The anesthetic level of CO_2 is well known to reduce the oxygen affinity and oxygen capacity of the blood. In the section I-1, the author showed the decrease in ATP content and energy charge value, and the increase in IMP content in the cold-CO_2 group (**Table I-1**). These findings supported the assumption that the carp in the cold-CO_2 group seemed to be in a substantial hypoxic condition despite the high level of partial pressure of O_2 in water (Po_2=650mmHg).

Table I-2. Initial values of [PCr]/[Pi], [ATP]/[Pi], pHi, and glycogen contents (mg/g) in carp muscle.

Group	[PCr]/[Pi]	[ATP]/[Pi]	pHi	glycogen
Control	1.67±0.21	0.51±0.04	6.9±0.1	10.5±1.1
Cold-CO_2	0.25±0.05*	0.24±0.04*	6.6±0.1*	7.1±0.9*

* Stastically significant at 5% level or better compared with control group. Values are expressed as mean±SD, n=10.

Significant differences were found in pHi and glycogen content between the control and cold-CO_2 groups. Anaerobic metabolism in fish is usually based on glycolysis (Van den Thillart and Van

Waarde, 1985), and the rate-limiting steps of glycolysis are those catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase. Normally, the glycolytic rate is mainly determined by the rates of hexokinase and phosphofructokinase while the pyruvate kinase keeps its step. Since phosphofructokinase activity has been shown to be pH-dependent (Fidelman et al., 1982) its activity should be affected by acute hypercapnia with its profound acidosis and accompanying inhibition of glycolysis. Concerning this phenomenon, Jakey and Schaefer (1972) reported that the blood phosphofructokinase activity in guinea pigs was inhibited by 55% when the blood pH was lowered by 0.4 as a result of hypercapnia (15% CO_2, 22% O_2, and N_2 balance). In this study, the glycogen still remained at 6.6mg/g in the muscle of the cold-CO_2 group. The muscle pH in the cold-CO_2 group was lower by 0.3 than that in the control group. The carp in the cold-CO_2 group seemed to fail in supply of high energy phosphates by glycolysis.

Postmortem Changes of ^{31}P NMR Spectra, pHi, and Glycogen Content of Carp Muscle

Figure I-5 shows the postmortem changes in ^{31}P NMR spectra of carp muscle of control and cold-CO_2 groups. In the control group, PCr decreased rapidly and then ATP decreased gradually. This indicates that the PCr acts as the energy reservoir for maintaining the ATP levels even in the muscle excised from fish as observed in the case of living fish muscle (Van den Thillart et al., 1989). The muscle PCr content was low in the cold-CO_2 group compared with that in the control group. This finding on the role of PCr in the muscle may partially explains the results that the

ATP-breakdown in the cold-CO_2 group was significantly faster than that in the control group (**Fig. I-2**).

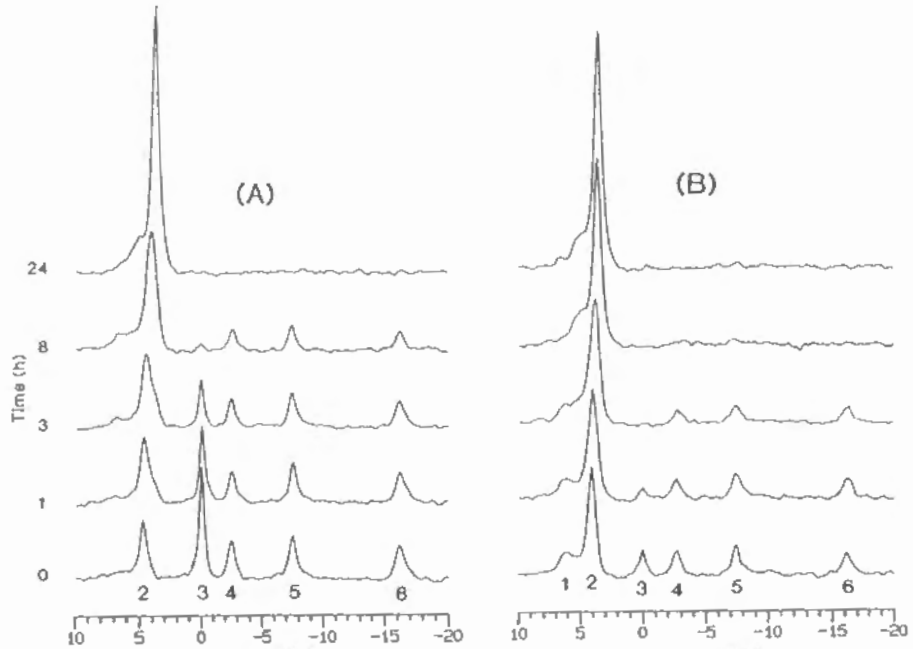

Fig. I-5 Postmortem changes of ^{31}P NMR spectra of carp muscle of control (A) and cold-CO_2 (B) groups after decapitation. Peak assignments are the same as those described in **Fig. I-4**.

A rapid decrease in the glycogen content was observed during 1 h postmortem in the control group (**Fig.I-6**), followed by a gradual decrease. On the other hand, the decrease in the glycogen content in the cold-CO_2 group during 1 h postmortem was much slow than that in the control group. The rate of the glycolysis seemed to be faster in the control group than in the cold-CO_2 group during 1 h postmortem. This may be one of the reasons for the difference in ATP degradation rate between the control and cold-CO_2 groups (**Fig. I-2**). In view of the faster rate of glycogen breakdown in the control group, we expected the rate of pH decline to be faster in

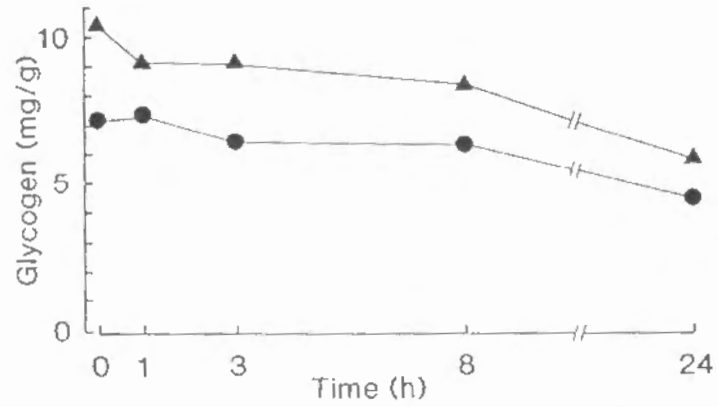

Fig. I-6 Changes in average glycogen content of carp muscle (n=5) during storage at 23° C. ▲, control group; ●, cold-CO_2 group.

the control group than in the cold-CO_2 group during 1 h postmortem. However, the rate of pHi decline hardly showed statistical differences between the control and cold-CO_2 during 1 h postmortem (**Fig I-7**). The exact reason for this phenomenon was not clear, but the buffering capacity of the muscle may be different in the two groups. These findings on pHi and glycogen along with the results about ATP and its related compounds indicate that the carp in the cold-CO_2 group was disturbed their energy state and muscle

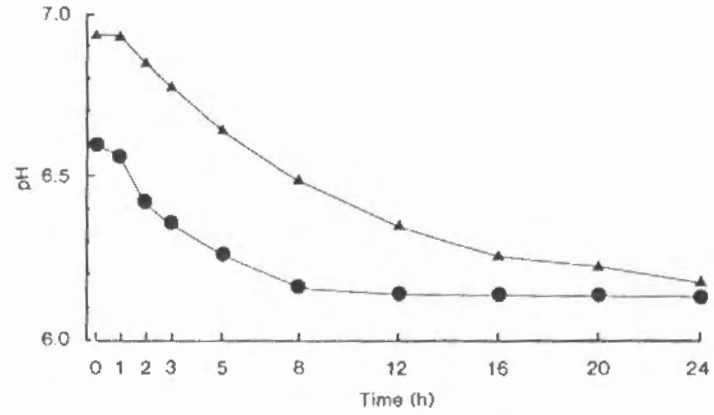

Fig. I-7 Changes in average pHi value of carp muscle (n=5) measured by ^{31}P NMR. ▲, control group; ●, cold-CO_2 group.

metabolism by the anesthetic stress. There would be difference in the onset of rigor-mortis and its development between the control and the cold-CO_2 groups, since there was differences in the levels and the changes of PCr and ATP content and pHi between the two groups (**Figs. I-2, I-5, and I-7**) (Iwamoto, 1991). The fact that changes in high energy phosphates as well as intracellular pH, can be measured repeatedly in the same muscle specimen by means of non-invasive ^{31}P NMR technique seems to provide fish meat scientists lots of merits. It should be interesting to estimate rigor mortis in relation to the data from NMR measurements.

Quality Evaluation of Carp Muscle by ^{31}P NMR

Figure I-8 shows the time courses of mean [PCr]/[Pi] and [ATP]/[Pi] ratios in carp muscle of control and cold-CO_2 groups.

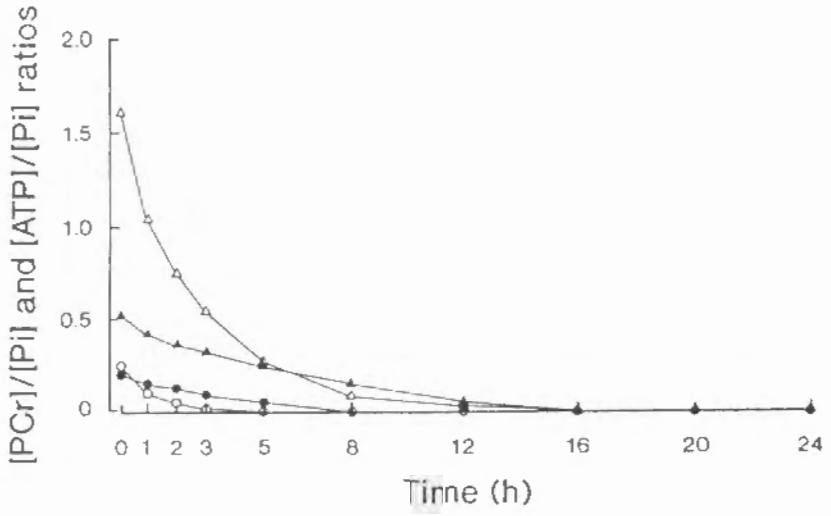

Fig. I-8 Changes in average [PCr]/[Pi] and [ATP]/[Pi] ratios of carp muscle (n=5) measured by ^{31}P NMR. △, [PCr]/[Pi] ratio of control group; ▲, [ATP]/[Pi] ratio of control group; ○, [PCr]/[Pi] ratio of cold-CO_2 group; and ●, [ATP]/[Pi] ratio of cold-CO_2 group.

The [PCr]/[Pi] ratio of control group decreased rapidly from 1.7 to 0.1 at 0 and 8 h storage, respectively. The [ATP]/[Pi] ratio of control group also decreased continuously during 16 h storage. On the other hand, the two ratios of cold-CO_2 group decreased continuously but those levels were very low. The specimen excised from both of the control and cold-CO_2 groups and kept in a plastic bag at 23° C, the same temperature of NMR measurement, emitted a faintly putrid smell at 8-10 h storage and the stage was recognized as the initial decomposition. For the evaluation of meat quality, such as freshness, the index must give information before the decomposition stage. In other words, the changing magnitudes of freshness index should be large during acceptable stage. The [PCr]/[Pi] ratio in the control group decreased rapidly and continuously during 8 h storage (**Fig. I-8**) and the magnitude of change was much large compared with the K value of control group (**Fig. I-3**). The [PCr]/[Pi] ratio seems to be suitable index for the evaluation of freshness in carp muscle which was not stressed before death. The levels of [PCr]/[Pi] and [ATP]/[Pi] were much different between the two experimental groups. Although the changes in the two ratios of cold-CO_2 group was very slow as in the case of K value, pHi measured by ^{31}P NMR decreased rapidly and continuously during storage (**Fig. I-7**). Judging from the several parameters obtained simultaneously by ^{31}P NMR, such as the ratios of high energy phosphates to Pi and pHi, it will be possible to evaluate meat quality such as freshness of carp muscle and also the energy state of the muscle including the fatigue of the fish i.e., whether the fish was stressed or unstressed before death. These results suggested that the ^{31}P NMR is a possible tool for the

evaluation of fish freshness, because of its non-invasive, convenient, rapid, and simultaneous determination of high energy phosphate compounds, PI, and pHi in the tissue of fish.

Chapter II. Postmortem changes of high-energy phosphate compounds in pelecypods

The utilization and consumption of marine mollusks are high in Japan, compared with those of other countries. A large amount of mollusk are consumed as fresh materials for a variety of dishes, including such popular dishes as sashimi and sushi. Consequently, the retention of freshness becomes a matter of serious concern, but the chemical assessment of freshness during storage of mollusk is less established than that for fishes. There seemed to be one reason that the postmortem degradation patterns of ATP have not been thoroughly investigated for mollusks being in good conditions (living in unstressed state). As for the degradation pathway of ATP, Saito (1961), Arai (1961a and 1961b), and Iwamoto et al. (1991) analyzed the muscle of several species of bivalves and reported that IMP did not accumulate during storage at -5° C and that ATP degradation proceeds as follows: ATP → ADP → AMP → AdR → HxR → Hx in surf clam Spisula sachalinensis, scallop Pecten yessoensis, and P. albicans, or ATP → ADP → AMP → AdR → Ad in ark shell Anadara broughtonii and abalone Haliotis discus, differing from fishes as reviewed by Saito (1961), and Uchiyama and Ehira (1970). On the other hand, Konosu et al. (1965) detected large amounts of IMP in edible parts of the short-neck clam Tapes japonica. There are some other reports that IMP, HxR, and Hx were detected during storage in the muscle of the scallop Placopecten magellnius (Hiltz and Dyer, 1970), oyster Crassostrea gigas (Suwetja et al., 1989; Sakaguchi et al., 1990), ark shell Anadara

broughtonii, clam Meretrix lusoria, and short-neck clam Tapes japonica (Suwetja et al., 1989). These results suggested the presence of the pathway, ATP → ADP → AMP → IMP → HxR → Hx. In those reports, there seem to be some problems, i.e. (1)ATP and its related compounds, especially IMP and AdR, was not detected simultaneously in each investigation. (2)Each specimen used in each investigation was dead one, commercially fresh one, or commercially alive one which was in much stressed state judging from the ATP level. (3)ATP and its related compounds were analyzed only in the muscle of each species. The postmortem patterns of ATP degradation in mantle, gill, or other tissues remains unclear. A species of which whole flesh is edible such as oyster, the patterns of ATP- degradation should be investigated in every tissue. (4)The degree of freshness of each specimen was not evaluated sensorially during storage. From these reasons, the chemical assessment of freshness during storage has not been established for bivalves.

In this chapter, the author developed the method for simultaneous determination of ATP and its related compounds, ATP, ADP, AMP, IMP, AdR, HxR, Hx, Xt, and Ad (Section II-1). Using this technique, the author examined the changes in levels of ATP and its related compounds in the adductor muscle, mantle, gill, and body trunk of the oyster, one of the most commercially important pelecypod in Japan, calculating K (Saito et al., 1959), K', and AEC values (Atkinson, 1968) from the levels of ATP and its related compounds in relation to the freshness of oysters (Section II-2). In the section II-3, the author showed the interesting effects of storage temperature on the postmortem ATP breakdown and efficacy

of freshness indices, K' and AEC values, at various storage temperature. In the section II-4, the author showed the activities of ATP-breakdown endogenous enzymes in oyster tissues and discussed the reasons for efficacy of K' and AEC values as for the freshness indices of oyster. Additionally, the author examined the efficacy of those chemical indices as for a freshness indicator of other species of pelecypods, ark-shell and hard clam (Section II-5).

In the chapter I, the ^{31}P NMR is shown to be a possible tool for evaluating fish meat quality, such as freshness, because of its non-invasive, convenient, rapid, and simultaneous determination of high energy phosphate compounds, PI, and pHi in the tissue of fish. Using this technique, the author examined non-invasively the postmortem changes of high-energy phosphate and evaluated the freshness of oyster (Section II-6).

II.1 Examination of the method for determining ATP and its related compounds in oyster by HPLC

The postmortem ATP-degradation in fish muscle proceeds as follows: ATP → ADP → AMP → IMP → HxR → Hx, as reviewed by Saito (1961) and Uchiyama and Ehira (1970). An HPLC method for simple reverse-phase separation with a commercially available column and for rapid and quantitative analysis of ATP and its breakdown products, has been established by Ryder (1985) and Tsuchimoto et al.(1985). The same postmortem pathway of ATP breakdown as revealed in fish was also suggested in the muscle of short-neck clam (Konosu et al., 1965), scallop (Hiltz and Dyer, 1970), oyster (Suwetja et al., 1989; Sakaguchi et al., 1990), ark shell, clam, short-neck clam (Suwetja et al., 1989). On the other hand, Saito (1961), Arai (1961a and 1961b) and Iwamoto et al.(1991) analyzed the muscles of several species of bivalves and reported that IMP did not accumulate during storage at -5° C and that ATP degradation proceeds as follows: ATP → ADP → AMP → AdR → HxR → Hx in surf clam, scallop or ATP → ADP → AMP → AdR → Ad in ark shell and abalone. However, they did not determine the content of AdR. They concluded the pathway of ATP degradation from the finding of little activity of AMP-deaminase in those bivalves. It seemed necessary to determine AdR to confirm the presence of this pathway in the shellfish tissues.

Among naturally occurring quaternary ammonium bases, large amounts of homarine (Suwetja et al., 1989; Gasteiger et al., 1960; Beers, 1967; Hirano, 1975) and trigonelline (Suwetja et al., 1989; Beers, 1967; Hiltz, 1970) are widely distributed in invertebrate

tissues. They affect the separation and quantitation of ATP and its related compounds in the tissue extract, since those two betaines which have a strong absorption at around 260-270nm often occur in tissue extracts. The HPLC system for the determination of ATP and its related compounds in invertebrates might enable to separate these compounds from the betaines. The author therefore developed a method for determining ATP and its related compounds, ATP, ADP, AMP, IMP, AdR, HxR, Hx, Xt, and Ad, and such 2 betaines as homarine and trigonelline, in shellfish by means of HPLC with isocratic elution. In this study, the author used oysters as a live specimen because of its easy handling.

Materials and Methods

Apparatus

Analyses were performed on an HPLC system, consisting of a Model 510 high-pressure pump, a Model 484 tunable absorbance detector and a Model HTR-B and C temperature control module (Waters chromatography Div. Millipore Corp., Osaka, Japan), a Model SIL-9A auto sample injector and a Model C-R4A computing integrator (Shimadzu, Kyoto, Japan). A CAPCELLPAK C18 SG column (4.6 x 150 mm, SHISEIDO, Tokyo, Japan) was used for the determination of ATP and its related compounds.

Reagents

The standards of ATP and its related compounds, ATP, ADP, AMP, IMP, AdR, HxR, Hx, Xt, and Ad, and trigonelline were purchased from Wako (Osaka, Japan). The standard of homarine was donated

from Prof. T. Hirano, Tokyo University of Fisheries. Other chemicals and solvents used were of analytical and chromatographic grade.

Specimen Preparation

Live cultured oysters were collected from a culture farm in Matoya Bay, Mie Prefecture. Whole oyster flesh was removed by cutting the adductor muscle close to the valves, and then lightly rinsed with a 1.5% ice-cold NaCl solution. Adductor muscle was dissected from the oyster fresh and stored for 2 days in a glass tube at 0° C. A 1-g portion of adductor muscle was homogenized with 5 ml of 10% perchloric acid (PCA), followed by centrifugation at 3,000 x g for 15 min. The residue was extracted twice with 4.5 ml of 5% PCA. The supernatants were combined and neutralized with 10 N and 1 N KOH solution on ice. The neutralized extract was centrifuged and the precipitate was washed with 10 ml of neutralized 5% PCA-KOH solution. The combined supernatants were diluted to 25 ml with distilled water. The PCA extract was filtered through a 0.22 μm microfilter. A 10-μl portion of the filtrate was then injected into the chromatographic system.

Chromatographic Conditions

The mobile phase for HPLC was a mixture of 20 mM citric acid, 20 mM acetic acid, and 40 mM triethylamine, pH 4.8. The solution was filtered through a 0.45 μm microfilter and degassed prior to use. The flow rate was 0.8 ml/min and the column temperature was held at 40° C. The eluate was monitored by UV absorption at 260 nm at the full scale of 0.02.

Determination of ATP and its related Compounds

ATP and its related compounds in the specimens were identified by comparison of retention time of peaks in HPLC between the specimens and the authentic compounds. For quantitation, the calibration curves were made with a peak-area in HPLC of the authentic compounds at various quantities.

Results and Discussion

Figure II-1 shows a chromatogram of 11 authentic compounds, ATP, ADP, AMP, IMP, AdR, HxR, Hx, Xt, Ad, trigonelline, and homarine, obtained by our newly improved HPLC system with isocratic elution. The ATP and its related compounds were effectively separated each other within 25 min. Homarine and trigonelline were eluted at 2.1

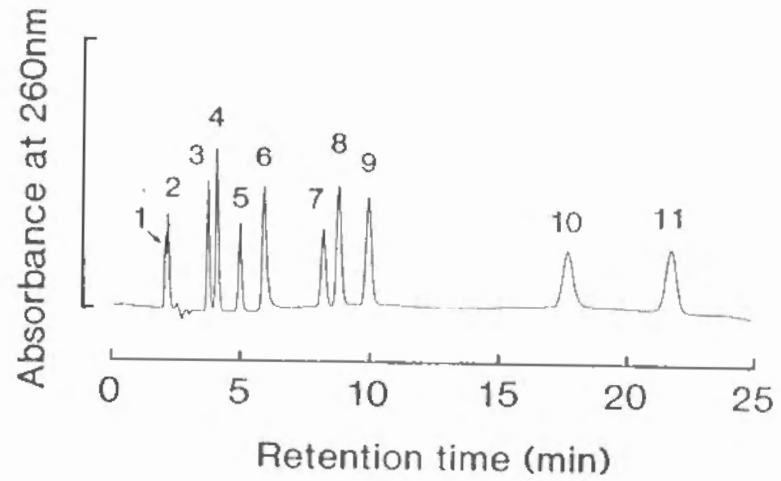

Fig. II-1 Chromatogram of standard solution of 9 authentic ATP and its related compounds, trigonelline, and homarine (each 0.1 nmol) by HPLC. 1, trigonelline; 2, homarine; 3, Hx; 4, Xt; 5, IMP; 6, Ad; 7,HxR; 8, AMP; 9, ADP; 10, ATP; and 11, AdR.

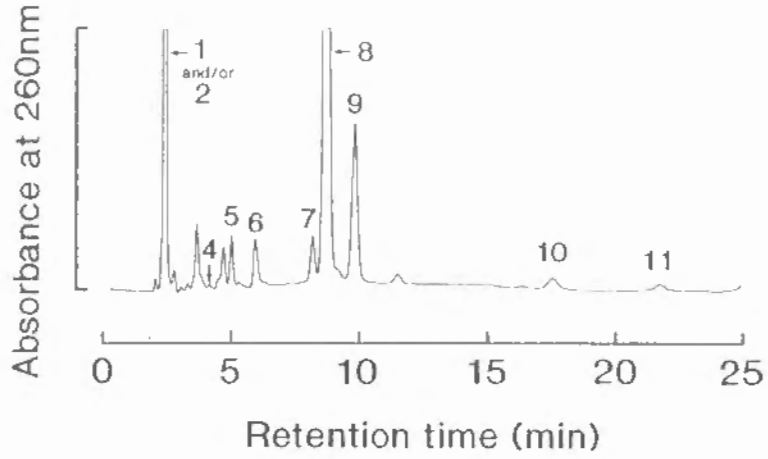

Fig. II-2 Chromatogram of the acid soluble fraction from the adductor muscle of oyster stored for 2 days in a glass vial at 0°C. Numbers on the HPLC peaks are the same as those given in Fig. II-1.

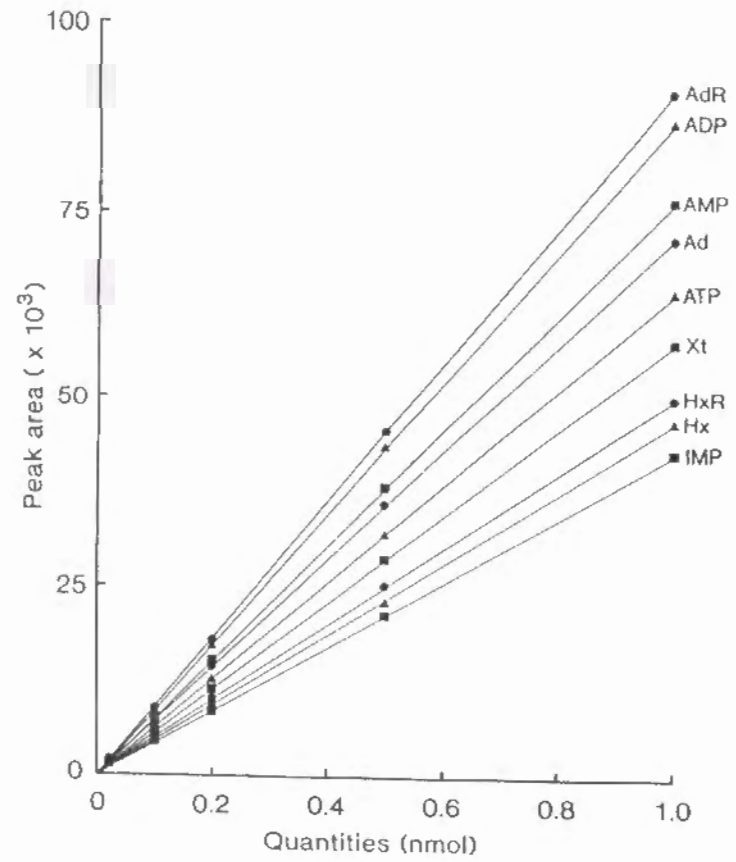

Fig. II-3 Calibration curves for estimating contents of ATP-related compounds.

and 2.25 min, respectively, and were not completely resolved. This lack of resolution was not a problem, since the two betaines did not affect the separation and quantitation of the ATP and its related compounds. **Fig.II-2** shows a chromatogram of the acid soluble fraction from the adductor muscle. In this case, the adductor muscle stored for 2 days in a glass tube at 0°C was used as the specimen for PCA extraction, since the AdR was absent in the time 0 specimen. We conducted to demonstrate the ability of our

Table II-1. Percentage recovery of ATP and its related compoundsadded to a PCA-muscle homogenate containing known initial concentration of the relevant compounds.

	recovery				
	amount added, μmol/g*				
compound	0.02	0.10	0.50	2.50	mean
ATP	92.8	104.0	97.0	98.1	98.0
ADP	110.3	95.9	94.6	93.6	98.6
AMP	94.1	101.2	94.3	97.2	96.7
IMP	107.0	93.7	89.8	93.7	96.1
AdR	98.3	94.6	102.7	98.3	98.5
HxR	101.4	96.5	89.8	92.7	95.1
Hx	104.1	97.0	93.8	96.7	97.9
Xt	106.3	95.8	94.7	97.6	98.6
Ad	90.7	103.0	97.0	100.3	97.8

*, PCA-muscle homogenate.

HPLC system to determine the AdR in the acid soluble fraction from oyster tissues. Large amounts of trigonelline and/or homarine were detected in the adductor muscle of oyster as reported in other invertebrates (Gasteiger et al., 1960; Beers, 1967; Hirano, 1975; Hiltz, 1970; Suwetja et al., 1989), but they did not affect the quantitation of the ATP and its related compounds in the acid soluble fraction from the oyster tissue as shown in **Fig.II-2**. The calibration curve for each compound was linear at least within the range of 0.01 to 1.0 nmol (**Fig.II-3**).

The recovery of ATP and its related compound added to a PCA-adductor muscle homogenate was good as shown in **Table II-1**. The mean recovery for each compound, over the range of amounts of added standard, ranged from 89.8% to 110.3 % with a mean recovery of each compound varying from 95.1% to 98.6%.

The author succeeded in measuring ATP and its related compounds and betaines. Using this technique, further investigation on the postmortem changes of ATP and its related compounds of mollusca was made in the following sections and chapters.

II.2 Postmortem changes of ATP and its related compounds and freshness indices in oyster tissues

Various pathways of ATP degradation have been reported in muscle tissues of several bivalves. Saito (1961), Arai (1961a and 1961b), and Iwamoto et al. (1991) analyzed the muscle of several species of bivalves and reported that IMP did not accumulate during storage at -5° C. ATP degradation reported to proceed as follows: ATP → ADP → AMP → AdR → HxR → Hx in surf clam and scallop, or ATP → ADP → AMP → AdR → Ad in ark shell and abalone, differing from fishes. However, they failed to detect AdR in the muscle of those bivalves. On the other hand, Konosu et al. (1965) detected large amounts of IMP in edible parts of the short-neck clam. There are other reports that IMP, HxR, and Hx were detected during storage in the muscle of scallop (Hiltz and Dyer, 1970), oyster (Suwetja et al., 1989; Sakaguchi et al., 1990), ark shell, clam, and short-neck clam (Suwetja et al., 1989). These results suggested the pathway, i. e. ATP → ADP → AMP → IMP → HxR → Hx. In those reports, ATP and its related compounds were analyzed only in the muscle of each species. The post-mortem patterns of ATP degradation in mantle, gill, or other tissues remains unclear. Furthermore, the chemical assessment of freshness during storage has not been established yet for bivalves.

In this section, the author describes the changes in levels of ATP and its related compounds in the adductor muscle, mantle, gill, and body trunk of the oyster Crassostrea gigas during ice storage using the HPLC method as described in Section II-1. Additionally, K (Saito et al., 1959), K', and AEC values

(Atkinson, 1968) were calculated from the levels of ATP and its related compounds in relation to the freshness of oysters.

Materials and Methods

Materials

Live cultured oysters were collected from a culture farm in Matoya Bay, Mie Prefecture. Oysters were artificially purified by placing them in filtered sea water sterilized by ultra-violet rays for 24h (Satoh, 1960). The most probable number (M.P.N.) of coli-group bacteria per 100g of oyster was effectively decreased from 2,200 to less than 7 after 24h of this treatment. After purification, the oysters were transferred within 4h to our laboratory in a container filled with sterilized sea water. Whole oyster flesh was removed by cutting the adductor muscle close to the valves, then lightly rinsing with ice-cold 1.5% NaCl. Each oyster was dissected into 4 tissues, adductor muscle, mantle, gill, and others (arbitrarily named body trunk), the average weights of which are shown in **Table II-2**. Each specimen of 4 tissues was held separately in a glass vial and stored in ice for 0, 1, 2, 4, 7, 10, 14, and 21days. At each fixed time of storage,

Table II-2. Fresh weight of the adductor muscle, mantle, gill, and body trunk (g, n=10, mean±S.D.).

Adductor muscle	Mantle	Gill	Body trunk	Total
2.01±0.35	3.56±0.56	2.23±0.14	4.16±0.67	11.96±0.44

five specimens of 4 tissues were frozen in liquid nitrogen, and stored at -85° C.

Preparation of Acid Soluble Fraction

A 1-g portion of each frozen sample was used for the preparation of acid soluble fraction as described in Section II-1.

Determination of ATP and its related Compounds by HPLC with Isocratic Elution

The PCA extract was filtered through a 0.22 μm membrane. A 10 μl portion of the filtrate was used to determine of ATP and its related compounds by HPLC as described in Section II-1.

Calculation of Chemical Freshness Indices

The K and K' values, the latter of which was named arbitrarily, were calculated from following equations:

$$K(\%) = (HxR+Hx)/(ATP+ADP+AMP+IMP+HxR+Hx) \times 100$$

$$K'(\%) = (IMP+HxR+Hx)/(ATP+ADP+AMP+IMP+HxR+Hx) \times 100$$

AEC value proposed by Atkinson (1968) as a metabolic regulatory parameter was calculated as a freshness index of oyster during storage from following equation:

$$AEC(\%) = 1/2\ (2ATP+ADP)/(ATP+ADP+AMP) \times 100$$

Sensory Evaluation

The degree of freshness of oyster tissues was evaluated by trained sensory panels and classified into three stages mainly based on its odor; acceptable (no smell), initial decomposition (faintly putrid smell), and advanced decomposition (putrid smell) as reported by Matsumoto and Yamanaka (1990).

Results

Changes in Content of ATP and Its Related Compounds of Oyster Tissues during Storage

Table II-3 shows the total amount of ATP and its related compounds at the start(0) and the end of storage (21 days). Adductor muscle contained approximately twice as much ATP and its related compounds as the other 3 tissues. The total content of ATP and its related compounds was relatively constant during storage(data not shown). At the end of storage, the levels in the adductor muscle, mantle, gill, and body trunk were 6.90, 2.78, 2.27, and 4.07(μmol/g wet tissue), respectively. The total contents of ATP-related compounds decreased only about 10% in each tissue after 21 days compared with the initial levels.

Table II-3. Total levels of ATP and its related compounds in oyster tissues (μmol/g wet tissue, n=5, mean±S.D.).

Storage time (days)	Adductor muscle	Mantle	Gill	Body trunk
0	8.23±2.16	3.70±0.83	2.64±0.45	4.87±0.84
21	6.90±1.89	2.78±0.90	2.27±0.36	4.07±0.76

Figure II-4 shows the changes in levels of ATP and its related compounds in the adductor muscle during ice storage. ATP levels fell from 3.57μmol/g at time 0 to 0.14μmol/g on the 1st day of storage. ADP decreased from 2.53 to 0.82μmol/g until the 2nd day and thereafter remained constant. On the other hand, AMP increased from 1.92 to 5.31μmol/g until the 1st day and decreased after 2

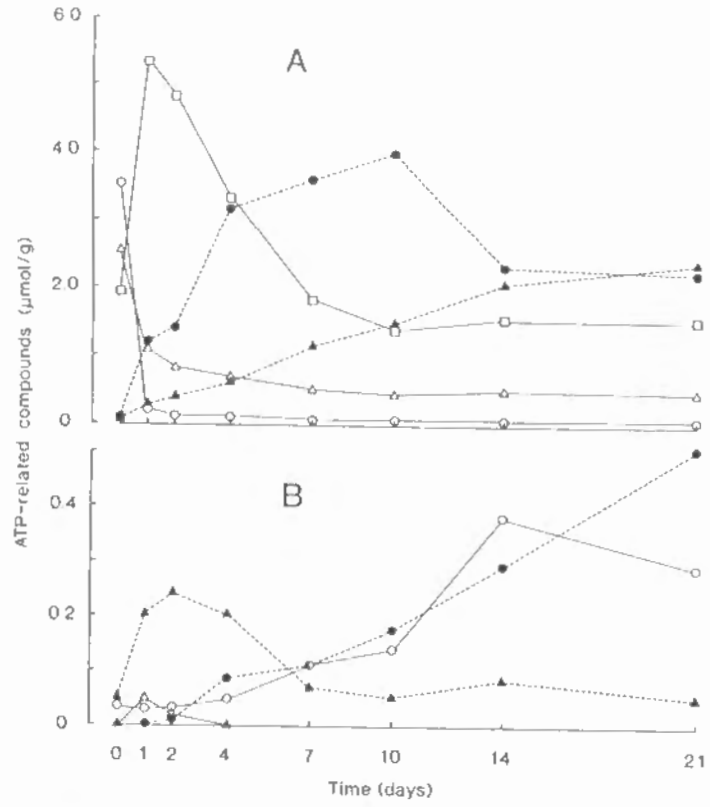

Fig. II-4 Changes in levels of ATP and its related compounds in the adductor muscle of oyster during ice storage.
A: ○, ATP; △, ADP; □, AMP; ●, IMP; and ▲, HxR.
B: ○, Hx; ●, Xt; △, AdR; and ▲, Ad.

days. IMP increased from 0.03 to 3.96μmol/g until the 10th day, and then decreased. AdR was detected only in the adductor muscle, increasing from 0μmol/g at time 0 to 0.05μmol/g on day 1, then disappearing after 4 days of storage. HxR increased from 0.07 to 2.37μmol/g during storage. Hx increased slowly until the 14th day, and then decreased. Xt increased slowly during storage. Ad increased slowly from 0.05 to 0.25μmol/g until the 2nd day, then decreased gradually.

In the mantle, ATP decreased linearly from 2.45 to 0.01μmol/g during storage (**Fig. II-5**). The decrease rate of ATP levels in the mantle was very low in contrast to that adductor muscle. The

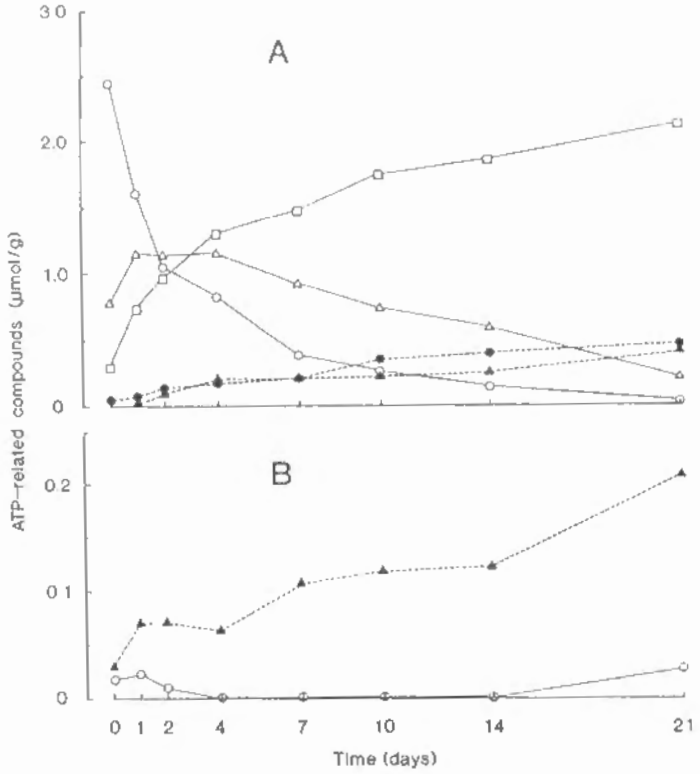

Fig. II-5 Changes in levels of ATP and its related compounds in the mantle of oyster during ice storage. Symbols in A and B are the same as those given in the footnote of Fig. II-4.

levels of ADP increased to 1.15μmol/g on day 1, and remained there until the 7th day, after which it decreased. AMP in the mantle increased linearly to 2.12μmol/g until the 21st day, unlike that in the adductor muscle. IMP increased linearly from 0.01 to 0.47μmol/g during storage. HxR increased slowly to 0.46μmol/g until day 21. Little amounts of Hx, AdR, and Xt were detected. the Ad levels were low, increasing slowly from 0.03 to 0.21μmol/g during storage.

In the gills, the changes in ATP and AMP reached 1.32μmol/g on the 10th day and then decreased gradually (**Fig. II-6**). IMP was constant at very low levels by day 2, and then increased linearly to 0.38μmol/g. AdR was undetectable, as observed in the mantle.

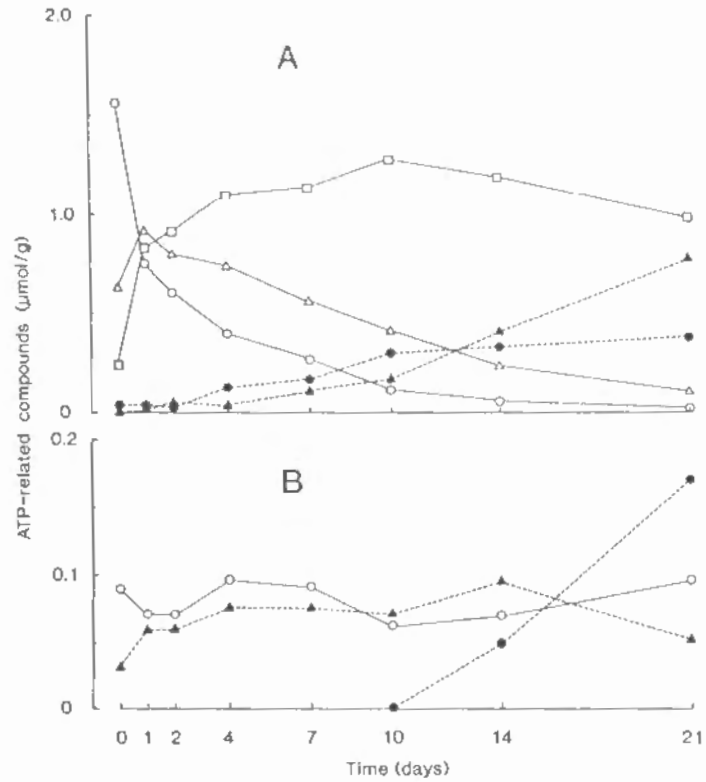

Fig. II-6 Changes in levels of ATP and its related compounds in the gill of oyster during ice storage. Symbols in A and B are the same as those given in the footnote of Fig. II-4.

HxR was at low levels until the 4th day, and then increased. Hx and Ad remained at low levels. Xt was detected on the 14th day, and then increased.

In the body trunk, ATP levels decreased linearly from 2.64 to 0.11μmol/g (**Fig. II-7**), and the rate was slower than those of either the mantle or the gill. ADP was constant until the 2nd day, and then decreased. The changes of AMP, IMP, and Ad were similar to those observed in the gill. AdR was undetectable. Hx increased from 0.01μmol/g on the 10th day to 0.37μmol/g on the 14th day, and thereafter decreased. On the other hand, Xt was detected for the first time on the 10th day, and then increased.

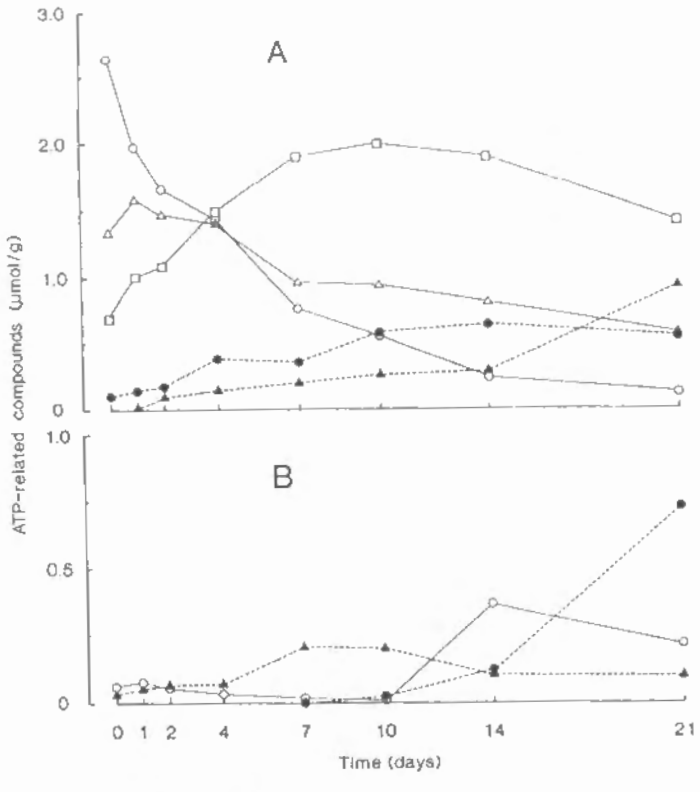

Fig. II-7 Changes in levels of ATP and its related compounds in the body trunk of oyster during ice storage. Symbols in A and B are the same as those given in the footnote of Fig. II-4.

Chemical Freshness Indices

Figure II-8 shows the changes in K, K', and AEC values together with sensory ratings in the adductor muscle during ice storage. On the 10th day of storage, the adductor muscle gave off a faintly putrid smell and this stage was recognized as the stage of initial decomposition. The K value increased slowly and linearly from 1.6 to 37.2% during storage. At the stage of initial decomposition the K value was 21.8%. The K' value increased from 1.9 to 75.1% by the stage of initial decomposition, and then remained constant. The AEC value fell from 60.3% at time 0 to 10.0% on the 1st day, after which it was then relatively constant.

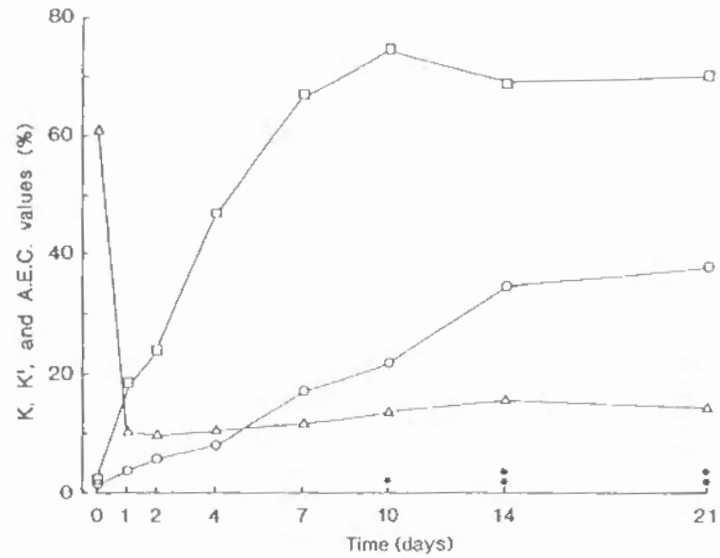

Fig. II-8 Changes in K, K', and AEC values, and sensory ratings in the adductor muscle of oyster during ice storage.
○, K value; □, K' value; and △, AEC value.
*, Initial decomposition; and **, advanced decomposition.

In the mantle, the K value increased slowly for 2 days, and then reached a stable level of 6-7% until the 14th day (**Fig. II-9**). The K value was only 14.1% at the initial decomposition stage on the 21st day. On the other hand, the K' value increased linearly from 3.0% at time 0 to 28.5% at the initial decomposition stage on the 21st day. The AEC value decreased rapidly from 80.3% at time 0 to 9.4% at the initial decomposition stage.

The K value of the gill increased slowly for 4 days, reaching a stable level of about 10% until the 10th day, the stage of initial decomposition, and then rose rapidly as the decomposition progressed (**Fig. II-10**). The K' value increased linearly and rapidly in comparison with the K value. The K' value was 22.1% at the stage of initial decomposition. The A.E.C. value decreased rapidly from 76.6% at time 0 to 17.8% at the initial decomposition

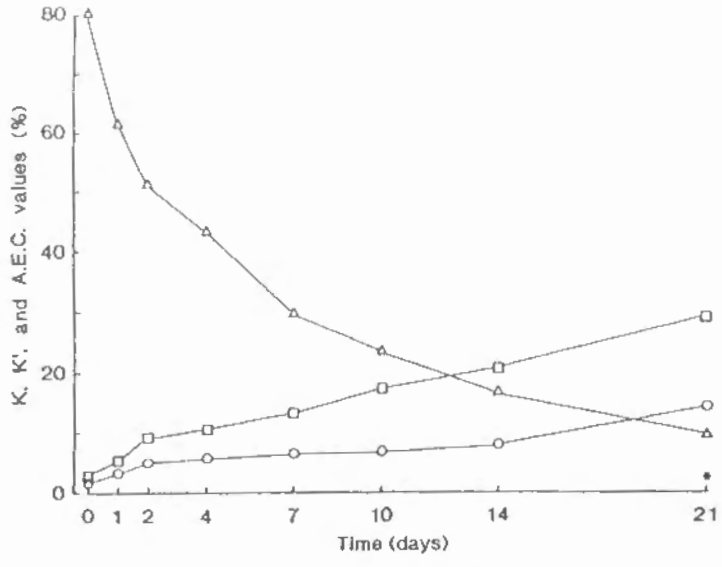

Fig. II-9 Changes in K, K', and AEC values, and sensory ratings in the mantle of oyster during ice storage. Symbols are the same as those given in the footnote of Fig. II-8.

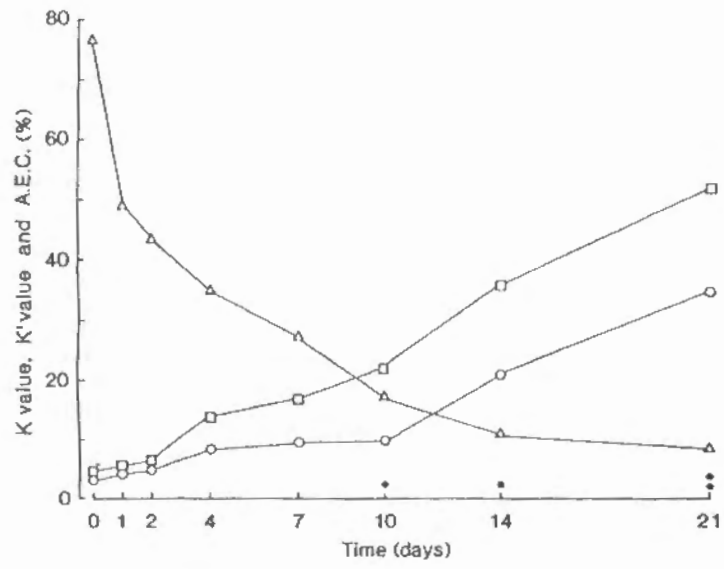

Fig. II-10 Changes in K, K', and AEC values, and sensory ratings in the gill of oyster during ice storage. Symbols are the same as those given in the footnote of Fig. II-8.

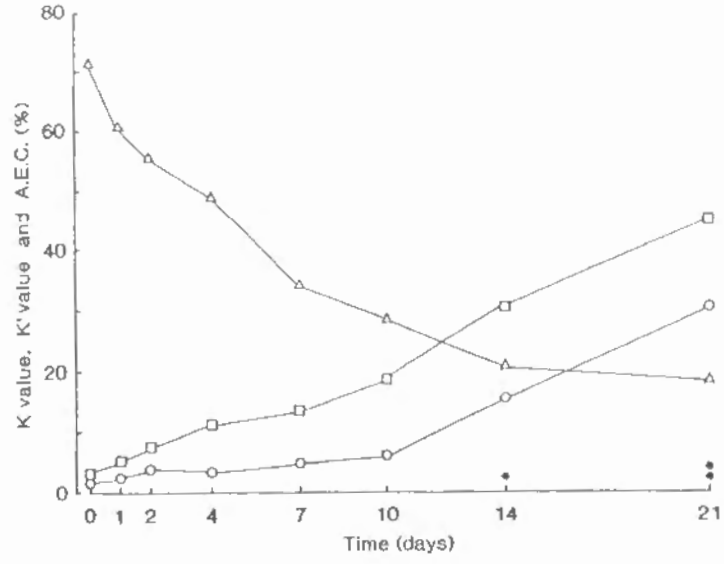

Fig. II-11 Changes in K, K', and AEC values, and sensory ratings in the body trunk of oyster during ice storage. Symbols are the same as those given in the footnote of Fig. II-8.

stage, then decreased slowly to 5.0% as decomposition progressed.

In the body trunk, the changes in K and K' values were similar to those of the gill; the initiation of decomposition was 4 days later (Fig. II-11). The A.E.C. value decreased rapidly from 71.0% at time 0 to 21.5% at the initial decomposition stage on the 14th day, and then decreased slowly.

Discussion

It has been reported that the patterns and rates of ATP degradation differ between dark and white muscle types of the yellowtail Seriola quinqueradiata (Murata and Sakaguchi, 1982). In this study, the total content of ATP and its related compounds in the adductor muscle was about 8.2μmol/g. This was much higher than those in the mantle, gill, and body trunk, which were about

3.7, 2.6, and 4.9μmol/g, respectively (**Table II-3**). The total content of ATP and its related compounds in the adductor muscle, about 8.2μmol/g, was higher than that (about 3μmol/g) in the muscle of the oyster reported by Suwetja et al. (1989). This may have resulted from physiological conditions of oysters, experimental conditions and/or the seasonal variation as reported by Watanabe et al. (1985) in the muscle of the ascidian Halocynthia roretzyi.

The degradation patterns of ATP and its related compounds also differed among the 4 oyster tissues as shown in **Figs. II-4, 5, 6, and 7**. In the adductor muscle, ATP decreased rapidly as found in the muscle of itayagai scallop Pecten albicans during storage at 0°C, by Iwamoto et al. (1991). ADP decreased slowly compared with fish muscle, and AMP accumulated as also reported in the muscle of marine invertebrates by Saito et al. (1958a, 1958b, and 1961), Arai (1961a and 1961b), and Iwamoto et al., (1991) but then decreased. On the other hand, ATP decreased rather slowly in the mantle, gill, and body trunk. ADP accumulated at first, then AMP, with the decrease of ADP as in the muscle during storage. The rates of changes in ATP, ADP, and AMP in the 3 tissues, however, were much slower than those in the adductor muscle. The enzyme systems (ATPase, myokinase, and AMP deaminase) responsible for the degradation of ATP, ADP, and AMP seemed to be highly active in the adductor muscle compared with those in the mantle, gill, and body trunk. Saito et al. (1958a, 1958b, and 1961), Arai (1961a and 1961b), and Iwamoto et al. (1991) analyzed the muscles of several species of shellfishes and reported that IMP did not accumulate

under the storage at -5 or 0°C. On the other hand, Suwetja et al. (1989) and Sakaguchi et al. (1990) detected the accumulation of IMP in the muscle of bivalves. In this study, the accumulation of IMP and HxR was also observed in the adductor muscle. In the mantle, gill, and body trunk, a small amount of IMP was also detected in the adductor muscle, but not in the mantle, gill, and body trunk. Ad was detected in all 4 tissues at much lower levels than IMP; thus, it can generally be considered that there are two or three pathways of AMP degradation (AMP → IMP → HxR, AMP → AdR → HxR, and/or AMP → AdR → Ad) in the adductor muscle of oyster. The IMP pathway was also present and seemed to be predominant in the mantle, gill, and body trunk during storage, judging by the IMP, AdR, Ad, and HxR accumulation. Although the activities of 5'-nucleotidase and both AMP and AdR deaminases were detected in the muscle, mantle, and hepatopancreas in shellfishes (Fujisawa and Yoshino, 1987; Lazou, 1989), the AdR pathway after death in the adductor muscle, mantle, gill, and body trunk of oyster was considered to be a minor pathway of AMP degradation during ice storage.

Table II-4 shows the changes in total AMP and IMP levels in the adductor muscle, mantle, gill, and body trunk, as well as the whole body, calculated from their fresh weight (g) (**Table II-2**) and the contents (μmol/g) of AMP and IMP (**Figs. II-4, 5, 6, and 7**) during storage before the stage of initial decomposition. AMP was detected at the level of 90mg% (about 2.6μmol/g), 172mg% (5.0μmol/g), and 32mg% (0.92μmol/g) in muscle extracts of abalone, scallop, and crab meat, respectively, and most probably act

synergistically with Glu to produce umami taste (Yamaguchi and Watanabe, 1988; Konosu et al., 1987). In this study, the average AMP contents of the whole body were 0.69-1.62μmol/g (**Table II-4**). In the oyster, AMP may be a taste-active component contributing to its meat taste. On the other hand, IMP shows a marked umami taste and is said to provoke a much higher umami sensation than AMP (Maga, 1983). Concerning this phenomenon, Komata (1964) detected 10.4mg/100g (0.30μmol/g) of AMP and 2.3mg/100g (0.07μmol/g) of IMP in sea urchin extract, and reported that IMP was a taste-active component that produced a meat flavor, but that AMP was not. Furthermore, Murata and Sakaguchi (1989) reported that 0.125μmol/ml of IMP in muscle extracts of yellowtail was enough to recognize the umami taste, 0.25μmol/ml increased the thickness intensity together with the umami, and 0.5μmol/ml increased all flavors (including umami, thickness, sourness, fresh fish flavor, and overall taste quality). Contents of IMP in the whole body were 0.06, 0.28, 0.33, 0.75, and 0.82μmol/g at 0, 1, 2, 4, and 7 days of storage, respectively (**Table II-4**). These results suggested that IMP plays an important role in producing umami and/or other flavor sensations in the oyster. Incidentally, AMP levels in the adductor muscle were much higher than those in the other 3 tissues at 0, 1, and 2 days of storage, and large amounts of IMP also accumulated in the adductor muscle (**Table II-4**). The taste of the adductor muscle, therefore, contributes to a comparatively large extent to that of the whole body of the oyster.

In the adductor muscle, HxR increased linearly and reached 1.13μmol/g on the 7th day of storage before the initial

Table II-4. Changes in total levels of AMP and IMP in the adductor muscle, mantle, gill, body trunk, and whole body (μmol/each part), or average levels of AMP and IMP in the whole body (μmol/g).

Storage time (days)		Total content (μmol/each part)				Average content
		Adductor muscle (μmol/2.01g)	Mantle (μmol/2.01g)	gill (μmol/2.01g)	Body trunk (μmol/2.01g)	Whole body (μmol/2.01g)	in whole body (μmol/g)
0	AMP	3.85	1.03	0.56	2.83	8.27	0.69
	IMP	0.06	0.14	0.07	0.45	0.72	0.06
1	AMP	10.67	2.67	1.85	4.20	19.39	1.62
	IMP	2.41	0.24	0.07	0.58	3.30	0.28
2	AMP	9.73	3.42	2.00	4.53	19.68	1.65
	IMP	2.69	0.50	0.04	0.71	3.94	0.33
4	AMP	6.71	4.56	2.43	6.16	19.86	1.66
	IMP	6.39	0.61	0.27	1.66	8.93	0.75
7	AMP	3.70	5.34	2.50	7.86	19.40	1.52
	IMP	7.22	0.74	0.33	1.46	9.75	0.82

decomposition occurred. In the mantle, gill, and body trunk, the increase of HxR was extremely slow before the initial decomposition stage. Tomioka and Endo(1984) reported that IMP-degrading enzyme activities in fish muscle differed among species and was pH-dependent. Those results suggested that the activities of 5'-nucleotidase in the mantle, gill, and body trunk were lower than that in the enzyme level and/or pH. Relatively high amounts of Hx and Xt were detected at the decomposition stage in the adductor muscle and body trunk (**Fig. II-4 and 5**). The increase in content of those substances might be due to the action of bacterial enzymes as reported by Matsumoto and Yamanaka (1991). Although Hiltz and Dyer (1973) reported that the Hx content was a potential freshness index for scallop adductor muscle, Hx and Xt levels seemed to be useful as decomposition indices in oysters. HxR, increasing linearly during storage, seemed to be more useful as a freshness indicator of the oyster adductor muscle than Hx.

As for the chemical freshness indices of the oyster during ice storage, K, K', and AEC values were calculated from the levels of ATP and its related compounds (**Figs. II-8, 9, 10, and 11**). In the adductor muscle, the K value increased slowly during storage, and was only about 22% at the initial decomposition stage (10th day). The K value in the adductor muscle, however, was useful as a freshness indicator because of its linear increase at the acceptable stage as suggested by Sakaguchi et al. (1990) in the muscle of the oyster C. gigas and Matsumoto and Yamanaka (1990) in that of the Kuruma prawn Penaleus japonicus. The K' value of the adductor muscle increased linearly and much more rapidly, reaching

about 70% at time initial decomposition stage, then remained constant thereafter. Our preliminary study indicates that the whole body stored in ice reached the initial decomposition stage after 10-14 days of storage, as did the adductor muscle alone stored in ice. From these results, it can be concluded that the K' value in the adductor muscle is much more useful as a freshness indicator of both the adductor muscle and whole body of oyster than the K value.

In the mantle, gill, and body trunk, the K value remained low at the acceptable stage and increased as the decomposition progressed (**Figs. II-9, 10, and 11**). The K values in the 3 tissues seemed to be useful not as freshness indices but as decomposition indices. In contrast, the K' values in the 3 tissues increased slowly but linearly at the acceptable stage and reached around 20% at the initial decomposition stage. The K' value, therefore, could be useful as a freshness indicator in the mantle, gill, and body trunk.

The AEC value in the adductor muscle dropped from 60 to 10% after 1 day of storage. On the other hand, the AEC values in the mantle, gill, and body trunk decreased continuously from 70-80 to 10-20% during the acceptable stage. As a result, the AEC value in the mantle, gill, and body trunk appeared to be a potential index of the freshness of oyster.

II.3 Post-mortem changes of ATP and its related compounds and freshness indices in oyster tissues at various temperatures

The author described the changes in content of ATP and its related compounds in various tissues of the oyster during storage in ice (Section II-2) and the differences in contents and degradation patterns of those compounds among the tissues and the two pathways of AMP degradation (IMP and AdR pathways). The author also proposed the K' and AEC (Atkinson, 1968) values as potential freshness indices of oyster during ice storage. An interesting effect of storage temperature on the ATP degradation, i.e. a faster ATP degradation at a lower storage temperature, has been observed in the muscle of fishes (Iwamoto et al., 1985, 1986, 1987, 1988, and 1990), prawn (Matsumoto and Yamanaka, 1990), and shellfishes (Iwamoto et al., 1991; Watanabe et al., 1992; Kawashima and Yamanaka, 1992). In those reports, ATP degradation was analyzed only in the muscle of each species. The temperature effects on the ATP degradation in other tissues, such as mantle, gill, and body trunk, remain unclear. Additionally, Iwamoto et al.(1991) reported that the freshness index, K value (Saito et al., 1959) for the adductor muscle of Itayagai scallop increased faster during storage at -3 and 0° C than at 5 and 10° C, while the muscle permitted the worst organoleptic evaluation when stored at 10° C. Kawashima and Yamanaka (1992) also reported the similar changes in K value at various storage temperatures in the adductor muscle of scallop. These results indicate that the K value is not suitable as a freshness index for the muscle of those shellfishes.

It is necessary to determine whether or not the K' and/or AEC values can be applied to the oyster as freshness indices at various temperatures.

In this section, the effects of storage temperature on the changes of ATP and its related compounds and on the changes of freshness indices, K, K', and AEC values, were examined in the adductor muscle, mantle, gill, and body trunk of the oyster Crassostrea gigas.

Materials and Methods

Materials

Live cultured oyster Crassostrea gigas was collected from a cultured farm in Matoya Bay, Mie Prefecture. They were artificially purified (Satoh, 1960) as described in Section II-2. Each oyster was dissected into 4 tissues, adductor muscle, mantle, gill, and body trunk. Four tissues of each specimen were held separately in a glass vial and stored at 0, 5, 10, 15, and 25° C. At each fixed time of storage, 10 specimens of 4 tissues were used for the preparation of acid soluble fractions in the same manner as described in Section II-1.

Determination of ATP and Its Related Compounds

ATP and its related compounds, i.e. ATP, ADP, AMP, IMP, AdR, HxR, Hx, Xt, and Ad, were determined by high performance liquid chromatography (HPLC) as described previously in Section II-1.

Calculation of Chemical Freshness Indices

The K (Saito et al., 1959), K', and AEC (Atkinson, 1968) values

were calculated from the contents of ATP and its related compounds from following equations as reported in the section II-2:

$$K\ (\%) = (HxR + Hx)/(ATP + ADP + AMP + IMP + HxR + Hx) \times 100$$

$$K'(\%) = (IMP + HxR + Hx)/(ATP + ADP + AMP + IMP + HxR + Hx) \times 100$$

$$AEC\ (\%) = 1/2\ (2ATP + ADP)/(ATP + ADP + AMP) \times 100$$

Organoleptic Test

The sensory ratings of the 4 tissues were evaluated using the organoleptic test (Matsumoto and Yamanaka, 1990) in the same manner as described in Section II-2.

Results

Changes in Content of ATP and Its Related Compounds of Oyster Tissues during Storage at Various Temperatures

Figure II-12 shows the changes in average content of ATP and its related compounds in the adductor muscle (n=10) stored at 0, 5, 10, 15 and 25°C. During storage at 0°C, the ATP level decreased rapidly from 3.57±0.38 μmol/g (mean±S.D.) at time 0 to 0.45±0.17 μmol/g after 8 h of storage. On the contrary, the ATP level decreased slowly during storage at 5°C, and became 1.44±0.21 μmol/g after 8 h of storage. The rate of decrease in the ATP level during the first 8 h of storage at 0°C was significantly higher than that at 5°C ($p<0.05$, Student's t-test). During storage at 10°C, the rate of decrease in ATP level was also lower than that at 0°C. The rate of ATP degradation during the first 8 h of storage was in the order of 25°C > 15°C > 0°C > 10°C > 5°C storage. The ADP level at time 0 was 2.53±0.31 μmol/g. Although the ADP level

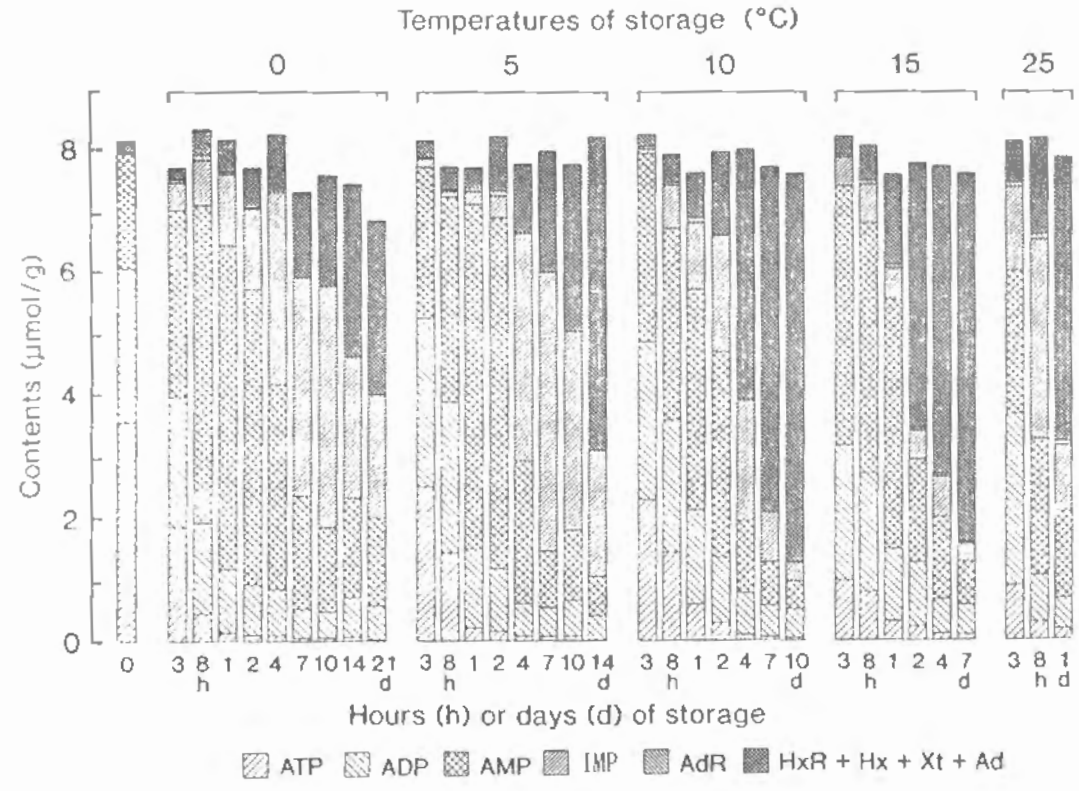

Fig. II-12 Changes in average content of ATP and its related compounds in the adductor muscle of oyster during storage at various temperatures (n=10).

decreased gradually during storage at all temperatures, the level after 8 h storage at 0°C, 1.47±0.19 μmol/g, was significantly lower than those at 5 and 10°C, 2.46±0.28 and 2.17±0.27 μmol/g, respectively ($p<0.05$). With decreasing in the ATP level, the levels of AMP and IMP increased markedly during storage at 0, 5, and 10°C. Additionally, the levels of AMP and IMP after 8 h storage at 0°C, 5.22±0.63 and 0.76±0.24 μmol/g, respectively, were significantly higher than those at 5°C, 3.38±0.27 and 0.11±0.04 μmol/g, respectively ($p<0.05$). The levels of IMP after 1 and 2 day storage at 0°C were also higher than those at 5°C ($p<0.05$). Although the level of AMP after 8 h storage at 10°C, 3.16±0.45 μmol/g, was lower than that at 0°C ($p<0.05$), the IMP level after

8 h storage at 10°C did not show the significant difference compared with that at 0°C. AdR was detected in small amounts during storage at all temperatures. The levels of HxR, Hx, and Xt increased rapidly during storage at higher temperatures. The total contents of ATP and its related compounds tended to decrease during storage at 0°C.

In the mantle, the ATP level decreased during storage (**Fig. II-13**). The rate of decrease in ATP level in the mantle was very low in contrast to that in the adductor muscle. The levels of ATP after

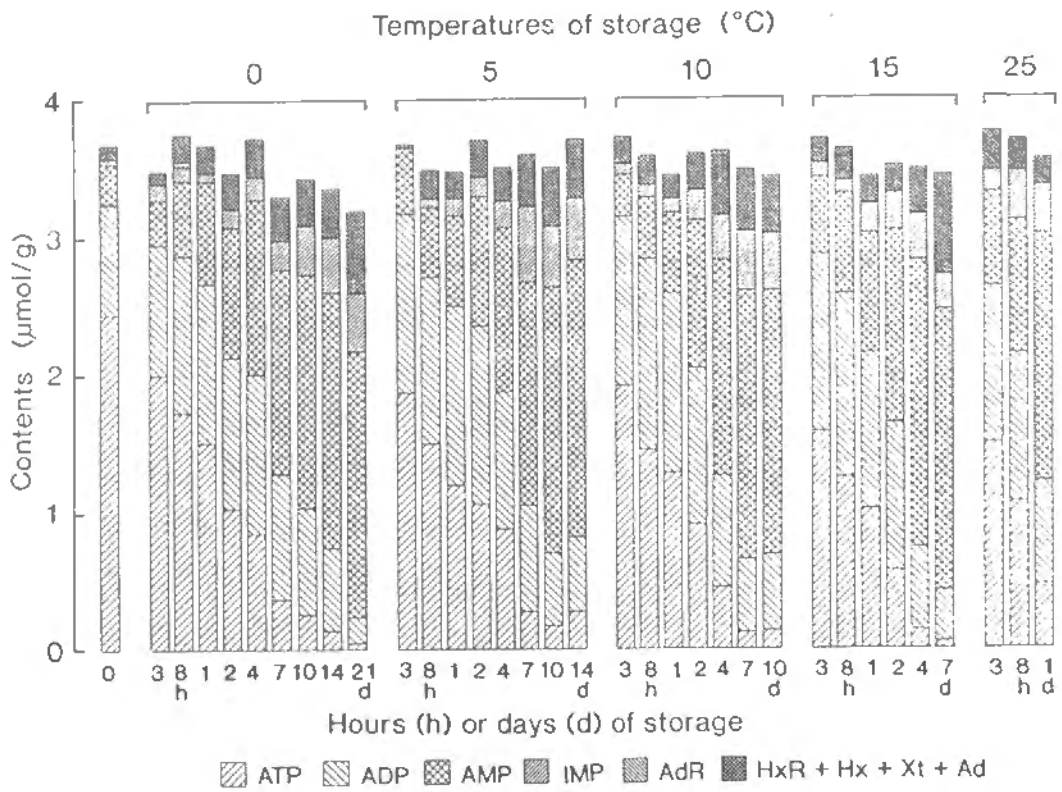

Fig. II-13 Changes in average content of ATP and its related compounds in the mantle of oyster during storage at various temperatures (n=10).

2 day storage at 0, 5, 10, and 15°C were 1.03±0.11, 1.06±0.13, 0.87±0.17, and 0.56±0.12 μmol/g, respectively. There was not a significant difference among those values at 0, 5, and 10°C during 2 days of storage ($P>0.05$), unlike those in the adductor muscle. The ATP level at 10°C, however, tended to decrease faster than those at 0 and 5°C ($P<0.1$). The rate of ATP degradation in the mantle during storage seemed to be in the order of 25°C > 15°C > 10°C > 5°C ≒ 0°C storage. The level of ADP increased at first, and then remained constant, after which it decreased during storage at

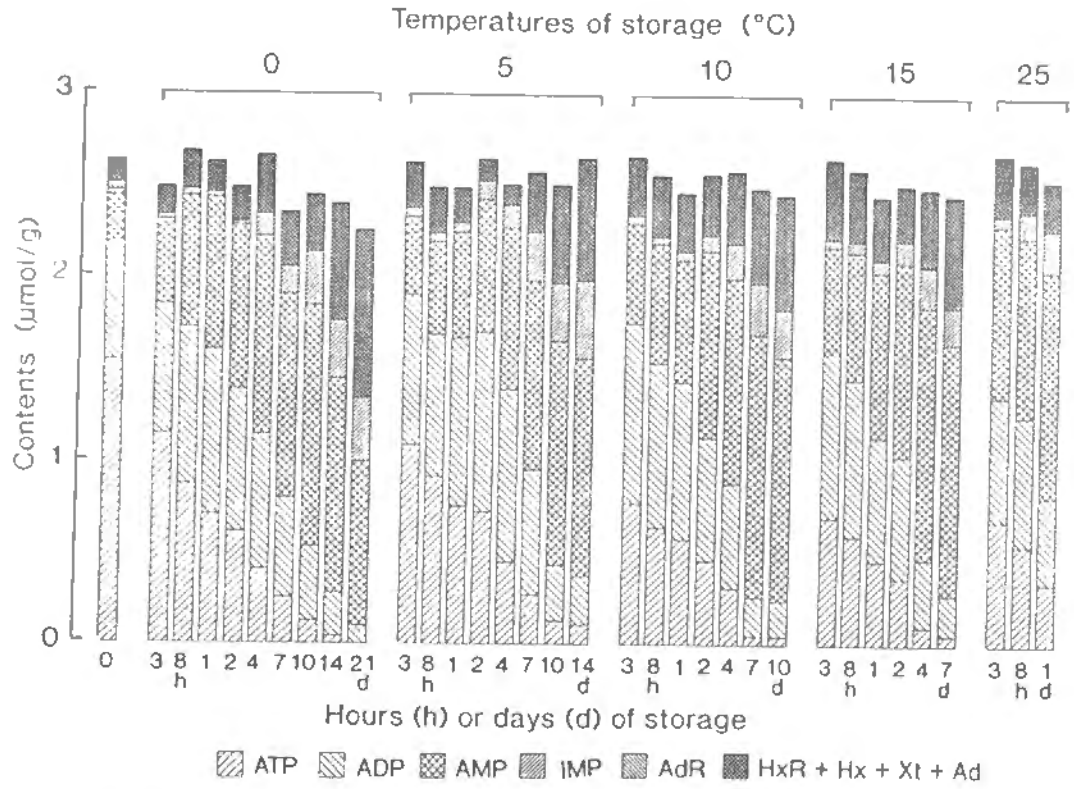

Fig. II-14 Changes in average content of ATP and its related compounds in the gill of oyster during storage at various temperatures (n=10).

all temperatures. The AMP level in the mantle increased to about 2μmol/g during storage. The levels of IMP, HxR, Hx, and Xt in the mantle were not as high as those in the adductor muscle. AdR was undetectable during storage at any temperature.

In the gill, the changes in ATP and ADP levels were similar to those in the mantle, and the rate of ATP degradation in the gill by the 1st day of storage was in the order of 25° C > 15° C > 10° C > 5° C ≒ 0° C storage (**Fig. II-14**). The IMP increased slowly during storage at all temperatures. AdR was undetectable, as in the

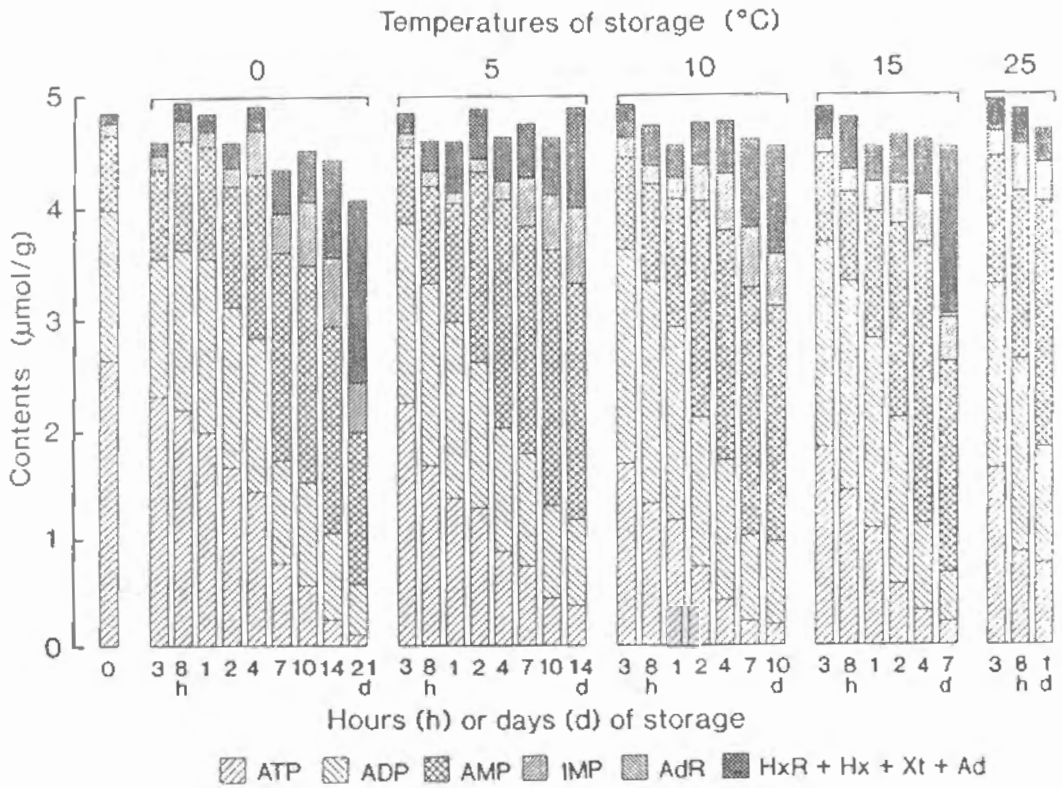

Fig. II-15 Changes in average content of ATP and its related compounds in the body trunk of oyster during storage at various temperatures (n=10).

mantle. The levels of HxR, Hx, Xt, and Ad in the gills increased slowly at a rate slightly higher than that in the mantle.

In the body trunk, the changes in levels of ATP and its related compounds during storage were similar to those observed in the gills (**Fig. II-15**), but the rate of ATP degradation in the body trunk during storage was in the order of 25° C > 15° C > 10° C > 5° C > 0° C storage. IMP was detected at a low level, while AdR was undetectable.

Chemical Freshness Indices

Figure II-16 shows the changes in K, K', and AEC values with sensory ratings obtained on the adductor muscle during storage at various temperatures. The adductor muscle gave a faintly putrid

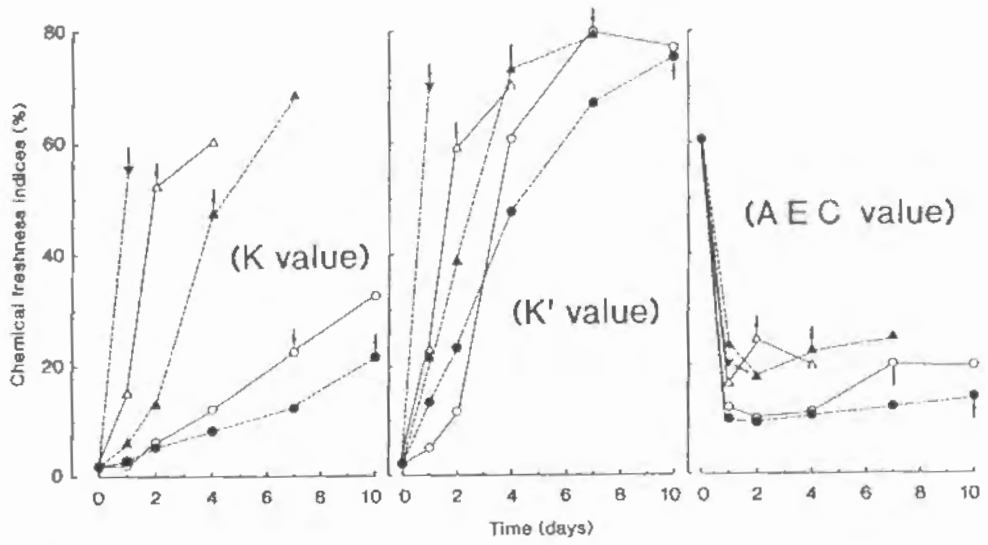

Fig. II-16 Changes in average K, K', and AEC values in the adductor muscle of oyster during storage at 0 (●), 5 (○), 10 (▲), 15 (△), and 25° C (▼) (n=10). Arrow indicates the stage of initial decomposition and thereafter the decomposition progressed.

smell on the 10th, 7th, 4th, or 2nd day of storage at 0, 5, 10, or 15° C, respectively, and this stage was recognized as the stage of initial decomposition. The K value increased very slowly during the acceptable stage at all storage temperatures, and then it increased rapidly as decomposition progressed. On the contrary, the K' value increased from 2% to about 60-80% by the stage of initial decomposition, and then remained constant. Although the K' values on the 1st and 2nd day of storage at 0° C were

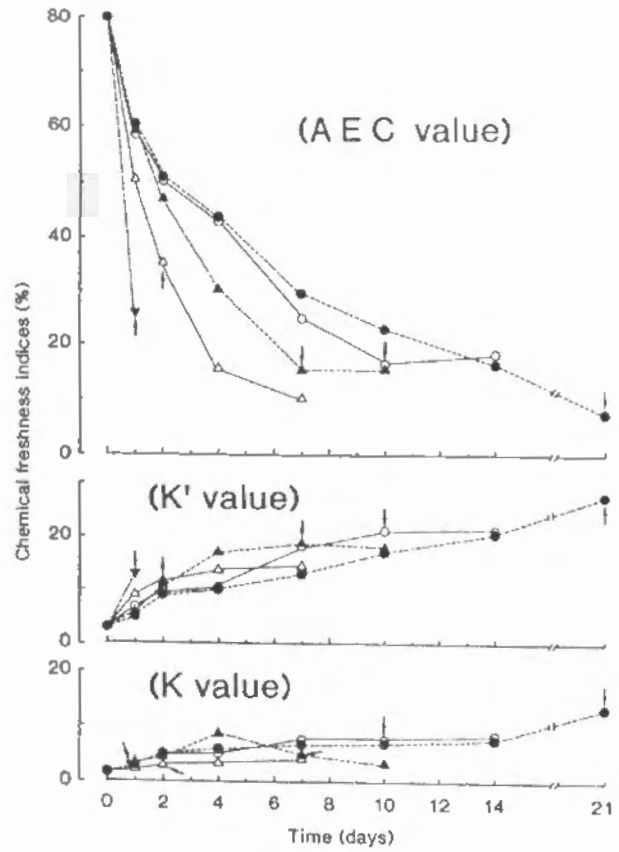

Fig. II-17 Changes in average K, K', and AEC values in the mantle of oyster during storage at 0-25° C (n=10). Symbols are the same as those given in the footnote of Fig. II-16.

significantly higher than those at 5° C ($P<0.05$) because of the higher levels of IMP at 0° C than those at 5° C (**Fig. II-12**), the K' value at 5° C was higher than that at 0° C on the 4th day of storage and thereafter. The AEC value fell from 60.3% at time 0 to about 10-20% on the 1st day. Thereafter the AEC value tended to increase because of the very low levels of ATP and the differences in rates between ADP and AMP decrease; the values were low compared with that at time 0.

In the mantle, the K value was very low during the acceptable stage (**Fig. II-17**). The K values was only from 2.7 to 13.9% at the initial decomposition stage. During storage at 10° C, the K value was 8.4% on the 4th day and then decreased as decomposition progressed. HxR and Hx increased until the 4th day, and then decreased. By way of compensation, Xt was detected for the first time on 4th day, and then increased. As a result, the K value decreased as the decomposition progressed at 10° C. On the other hand, the K' value increased from 3% at time 0 to about 20-30% at the initial decomposition stage during storage at 0, 5, or 10° C. The K' values increased during 15° C and 25° C storage; the K' values at the initial decomposition stage were only 10.7 and 13.4%, respectively. The AEC value decreased rapidly and continuously from 80.3% at time 0 during storage. The AEC values at the initial decomposition stage were slightly high, 36.2% and 27.7% , during storage at 15 and 25° C, respectively.

The K value obtained on the gills was low at the acceptable stage (**Fig. II-18**), and then rose rapidly as decomposition progressed at 0 and 5° C. The K' value increased linearly and rapidly compared with the K value during storage at 0, 5, and 10° C.

However, at 15 and 25° C, the K' value did not show a marked increase. The AEC value in the gills decreased rapidly and continuously during storage as observed in the mantle.

In the body trunk, the changes in K and K' values during storage were similar to those in the gills; a marked increase of K' value

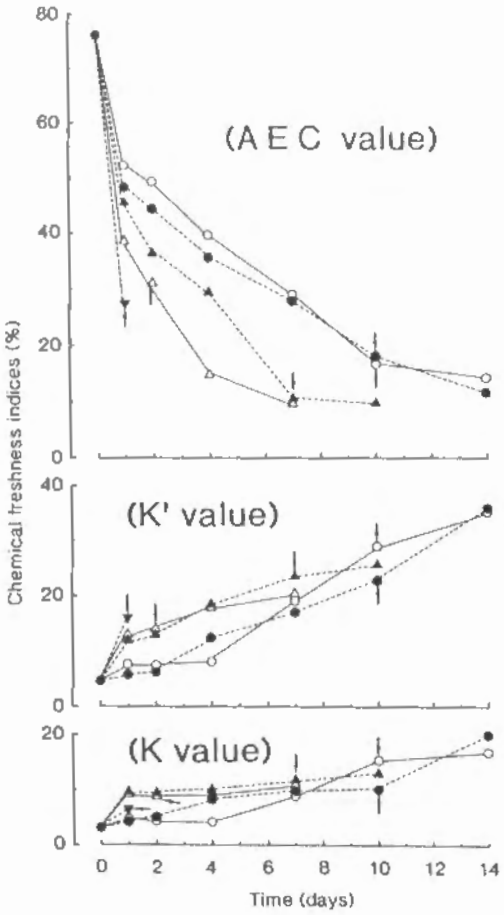

Fig. II-18 Changes in average K, K', and AEC values in the gill of oyster during storage at 0-25° C (n=10). Symbols are the same as those given in the footnote of **Fig. II-16.**

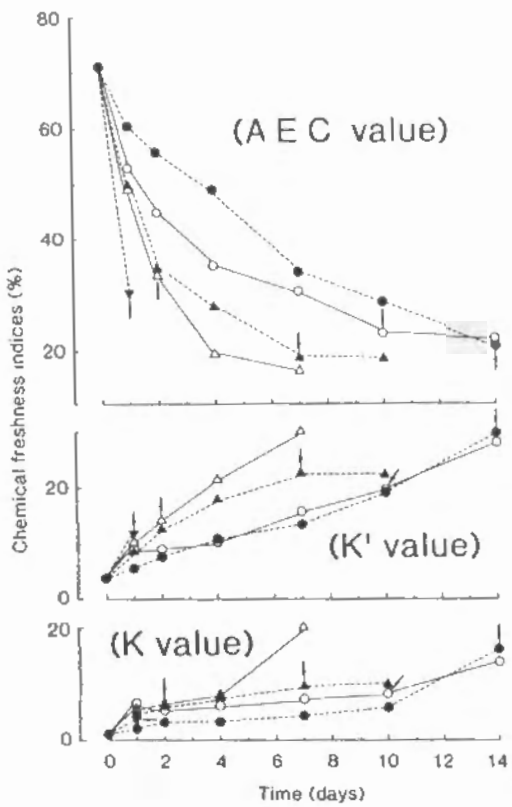

Fig. II-19 Changes in average K, K', and AEC values in the body trunk of oyster during storage at 0-25° C (n=10). Symbols are the same as those given in the footnote of **Fig. II-16.**

was observed at the decomposition stage at 15° C (**Fig. II-19**). The AEC value decreased rapidly from 71% at time 0 to about 20-35% at the initial decomposition stages at various storage temperatures.

Discussion

The author previously described that the total content of ATP and its related compounds differed with the oyster tissues, such as adductor muscle, mantle, gill, and body trunk in Section II-2. Watanabe et al. (1993) also reported similar results that the content of ATP and its related compounds were different among the disk abalone tissues including adductor muscle, foot muscle, mantle, and viscera. In this investigation, the author confirmed the previous findings that the total content of ATP and its related compounds in the adductor muscle was much higher than those in the mantle, gill, and body trunk of oyster (**Figs. II-12, 13, 14, and 15**). The total contents of ATP and its related compounds in oyster tissues tended to decrease during storage at 0° C. Although the exact reason of the phenomenon was unclear, one of the possible reasons was that the higher efflux of body fluid from the tissues because of the lower temperature and longer period of storage. The degradation pattern of ATP and its related compounds differed among the 4 tissues. The rate of ATP degradation in the adductor muscle was in the order of 0° C > 10° C > 5° C storage. Faster ATP degradation at a lower storage temperature has also been observed in the muscle of red sea bream (Iwamoto et al., 1985, 1986, and 1990), plaice (Iwamoto et al., 1987 and 1988), kuruma prawn (Matsumoto and Yamanaka, 1990),

itayagai scallop (Iwamoto et al., 1991), disk abalone (Watanabe et al., 1992), and scallop (Kawashima and Yamanaka, 1992). This phenomenon was reported in fish muscle by Watabe et al. (1989) and the same mechanism proposed by them would be applicable to the adductor muscle of oyster. On the other hand, the rate of ATP degradation in the body trunk was in the order of 10° C > 5° C > 0° C storage. Although the temperature effects on the ATP degradation in the body trunk was different from those in the adductor muscle, the exact reason of this disagreement is not clear. The temperature effects on the rates in ATP degradation during storage in the gill and mantle were intermediate between those in the adductor muscle and body trunk. The rates of changes in ATP, ADP, and AMP in the mantle, gill, and body trunk were generally much slower than those in the adductor muscle at each storage temperature, 0, 5, 10 ,15, and 25° C. The enzyme systems responsible for the degradation of ATP, ADP, and AMP, that is ATPase, myokinase, and AMP deaminase, respectively, seemed to be highly active in the adductor muscle compared with those in the other 3 tissues as described previously (Section II-2). The occurrence of AdR and IMP was confirmed with enzymatic analysis by Kawashima and Yamanaka (1992) in scallop muscle. In this study, at all storage temperatures, IMP and AdR were detected in the adductor muscle. Obviously, AMP was degraded in the adductor muscle of oyster through the two pathways as mentioned previously (Section II-2). In the other 3 tissues at various storage temperatures, however, IMP but not AdR was detected. The IMP pathway of AMP degradation seemed to be predominant during storage in these 3 tissues.

For the possible freshness indices of oyster, K, K', and AEC values were calculated from the levels of ATP and its related compounds (**Figs. II-16, 17, 18, and 19**). Iwamoto et al. (1991) reported the faster degradation of ATP and the faster increase in K value at a lower storage temperature in itayagai scallop. Similar changes in K value were also reported in scallop muscle by Kawashima and Yamanaka (1992). They concluded that the K value could not be applied as a freshness index to scallop. On the contrary, in the oyster adductor muscle, the faster increase in K value at lower storage temperatures was not detected, since AMP and IMP were accumulated rapidly and further degradation to HxR and Hx was not observed at the beginning of storage in the oyster muscle, unlike in the scallop muscles (Iwamoto et al., 1991; Kawashima and Yamanaka, 1992). Although the K value increased with storage time, its increasing rate during the acceptable stage was slow in the adductor muscle. The K value in the mantle, gill, and body trunk showed little increase and/or fluctuated with increasing storage temperatures. The K value was not suitable for oyster as a freshness index. On the other hand, the K' value in the adductor muscle increased rapidly from the first day of storage, reaching 70-80% at the initial decomposition stage, thereafter it remained constant during storage at 0, 5, and 10° C. At higher storage temperatures, 15 and 25° C, the K' value reached approximately 60% at the initial decomposition stage. The index for freshness must give information on freshness before the onset of decomposition as described by Watanabe et al. (1992) on disk abalone muscle. Therefore, the K' value obtained on the adductor muscle appeared to be useful as a freshness indicator of oyster

muscle because of its rapid and continuous increase throughout the acceptable stage. From the same point of view, the AEC values in the mantle, gill, and body trunk that showed a rapid and continuous decrease during the storage at all temperatures appeared to be useful as a freshness indicator for oyster.

II.4 ATP-breakdown by endogenous enzymes in oyster tissues

Postmortem changes in ATP and its related compounds in invertebrates are known to be different from those in vertebrates (Saito et al., 1958a and 1958b; Arai, 1961a and 1961b). In the previous section (Sections II-2 and II-3), the author showed that the postmortem changes of ATP and its related compounds in the oyster differed markedly from those in fishes and also were different among the tissues. In the adductor muscle, ATP decreased rapidly, while AMP and IMP accumulated. The K value increased linearly but slowly during storage. On the other hand, in the mantle, gill, and body trunk, ATP levels decreased slowly, whereas ADP and AMP accumulated. IMP was present at low levels. The K values in the 3 tissues were low at acceptable stage. From these results, the author discussed that the enzyme systems responsible for the degradation of ATP and its related compounds seemed to be highly active in the adductor muscle compared with those in the other tissues.

In this section, to clarify the role of endogenous enzymes and bacterial enzymes in the postmortem changes of ATP and its related compounds of oyster tissues, the effects of an antibiotic chloramphenicol on these changes were investigated.

Materials and Methods

Materials

Live cultured oysters (n=100) Crassostrea gigas were collected from a culture farm in Made bay, Mie prefecture. They were

artificially purified by placing them in filtered sea water sterilized by ultra-violet rays for 24 h (Satoh, 1960). Each oyster was dissected into 4 tissues, adductor muscle, mantle, gill, and body trunk. Each tissue was chopped into small pieces and made into composite samples. The composite sample of each tissue was divided into 4 portions, and 0.1% chloramphenicol (CP) was added to 2 portions. Each portion with and without CP was then homogenized. The portions of each tissues consisted of chopped and homogenized portions with and without CP then being stored at 5° C. The temperature was selected from the following reason; an interesting effect of storage temperature on ATP degradation, i.e. a faster ATP degradation at a lower storage temperature (25 > 15 > 0 > 10 > 5° C in the adductor muscle and 25 > 15 > 10 > 5 = 0° C in the mantle and gill) has been observed as shown Section II-3. The lowest temperature at that the interesting effects of storage temperature was not detected was 5° C. At each time of storage, tissue was withdrawn from each portion and subjected to the following tests.

Preparation of Acid Soluble Fraction

About 5 g of tissue was homogenized with 10 ml of ice-cold 10% perchloric acid, followed by centrifugation at 3,000 x g for 15 min. The procedure was repeated and the supernatants were combined. After neutralization with 10 N and 1 N KOH on ice, the neutralized extract was centrifuged and the supernatant was made up to 25 ml with distilled water. This solution was used for the determination of ATP and its related compounds.

Determination of ATP and Its Related Compounds

The contents of ATP and its related compounds were analyzed by HPLC using the method described in Section II-1.

Calculation of Chemical Freshness Indices

The K (Saito et al., 1959), K', and AEC (Atkinson, 1968) values were calculated from the contents of ATP and its related compounds from following equations as described in Section II-2:

$$K\ (\%) = (HxR + Hx)/(ATP + ADP + AMP + IMP + HxR + Hx) \times 100$$

$$K'(\%) = (IMP + HxR + Hx)/(ATP + ADP + AMP + IMP + HxR + Hx) \times 100$$

$$AEC\ (\%) = 1/2\ (2ATP + ADP)/(ATP + ADP + AMP) \times 100$$

Organoleptic Test

The sensory ratings of the 4 tissues were evaluated using the organoleptic test (Matsumoto and Yamanaka, 1990) in the same manner as described in Section II-2.

Results

Table II-5 shows the changes in ATP and its related compounds and freshness indices together with sensory ratings in the chopped adductor muscle with and without CP during storage at 5° C. Portions of chopped adductor muscle without CP were judged at the stage of initial and advanced decomposition on the 4th and 7th day of storage, respectively. For portions with CP, on the contrary, little decomposition was detected during storage. The changes in ATP, ADP, and AMP levels in the chopped adductor muscle without CP were similar to those with CP. ATP decreased rapidly for 1 day of storage, ADP decreased gradually during storage, and AMP increased

Table Ⅱ-5. Influence of chloramphenicol (CP) on the contents of ATP and its related compounds and freshness indices in the chopped adductor muscle during storage at 5℃.

	Storage time (days)	ATP-related compounds (μmol/g)										Freshness indices (%)			Sensory ratings*
		ATP	ADP	AMP	IMP	AdR	HxR	Hx	Ad	Xt	Total	K	K'	AEC	
Without CP	0	2.16	1.44	1.61	0	0	0.03	0.02	0.03	0	5.29	1	1	55	1
	1	0.39	0.80	1.87	1.09	0.03	0.14	0.01	0.18	0.02	4.52	3	28	26	1
	2	0.21	0.62	1.39	1.75	0.03	0.31	0.03	0.21	0.04	4.58	8	48	26	1
	4	0.17	0.42	0.60	0.93	0.03	1.17	0.31	0.11	0.15	3.89	41	67	32	2
	7	0.14	0.23	0.36	0.13	0.02	0.12	2.38	0.02	0.53	3.92	74	78	35	3
	10	0.14	0.30	0.32	0.13	0	0.07	2.33	0.02	0.94	4.25	73	77	38	3
With CP	0	2.16	1.44	1.61	0	0	0.03	0.02	0.03	0	5.29	1	1	55	1
	1	0.24	0.51	2.44	0.89	0.04	0.18	0.02	0.23	0.02	4.57	5	26	15	1
	2	0.14	0.37	1.47	1.76	0.02	0.35	0.05	0.29	0.07	4.53	10	52	16	1
	4	0.15	0.23	0.52	2.51	0	0.57	0.18	0.22	0.17	4.54	18	78	29	1
	7	0.09	0.16	0.17	2.31	0	0.88	0.33	0.11	0.34	4.39	31	89	39	1
	10	0.10	0.22	0.16	1.73	0	1.12	0.53	0.10	0.43	4.37	43	88	44	1

*; 1, Acceptable; 2, Initial decomposition; 3, Advanced decomposition.

Table Ⅱ-6. Influence of chloramphenicol (CP) on the contents of ATP and its related compounds and freshness indices in the homogenized adductor muscle during storage at 5℃.

	Storage time (days)	ATP-related compounds (μmol/g)										Freshness indices (%)			Sensory ratings*
		ATP	ADP	AMP	IMP	AdR	HxR	Hx	Ad	Xt	Total	K	K'	AEC	
Without CP	0	2.16	1.44	1.61	0	0	0.03	0.02	0.03	0	5.29	1	1	55	1
	1	0.02	0.30	2.40	1.03	0	0.45	0.01	0.10	0.04	4.34	11	35	6	1
	2	0.01	0.27	1.42	1.88	0	0.74	0.07	0.12	0.09	4.58	18	61	9	1
	4	0.03	0.22	0.11	0.14	0	0.22	2.65	0.01	0.25	3.63	85	89	39	2
	7	0	0.13	0.02	0.01	0	0.05	3.17	0	0.60	3.98	95	95	42	3
	10	0	0.07	0.01	0	0	0.07	3.54	0	0.74	4.41	98	98	46	3
With CP	0	2.16	1.44	1.61	0	0	0.03	0.02	0.03	0	5.29	1	1	55	1
	1	0.03	0.31	2.56	0.99	0	0.44	0.01	0.11	0.02	4.45	10	33	6	1
	2	0.02	0.27	1.53	1.84	0	0.71	0.04	0.13	0.07	4.61	17	59	9	1
	4	0.01	0.24	0.26	2.71	0	1.01	0.23	0.11	0.19	4.75	28	89	25	1
	7	0	0.21	0.71	2.13	0	1.31	0.54	0.09	0.32	5.30	38	81	11	1
	10	0	0.19	0.03	1.25	0	1.41	0.85	0.08	0.36	4.16	61	94	43	1

*; 1, Acceptable; 2, Initial decomposition; 3, Advanced decomposition.

first and then decreased gradually. Small quantities of AdR were detected both in the muscle with and without CP. On the other hand, the changes in IMP, HxR, Hx, and Xt levels in the chopped adductor muscle without CP differed from those with CP. In the chopped adductor muscle without CP, at first IMP and HxR increased and then decreased as the decomposition progressed. Hx increased slowly during acceptable stage and rapidly as the decomposition progressed. In those with CP, IMP increased for 4 days of storage and remained at high levels. HxR increased continuously during storage. The increasing rates of HxR, Hx, and Xt in the muscle with CP were much slow compared with those without CP. The increasing rate of K value in the muscle without CP was faster than that with CP especially at the stage of decomposition. On the other hand, the change in K' value in the muscle without CP was similar to that with CP.

Table II-6 shows the changes in ATP and its related compounds and freshness indices together with sensory ratings in the homogenized adductor muscle with and without CP during storage at 5° C. Although the rates of ATP decrease and Hx increase in the homogenized muscle were a little faster than those in the chopped muscle, generally the changes and their rates of ATP and its related compounds in the homogenized adductor muscle with and without CP were similar to those in the chopped muscle.

Table II-7 and 8 show the changes in ATP and its related compounds and freshness indices together with sensory ratings in the chopped and homogenized mantle, respectively, with and without CP during storage at 5° C. Portions of both chopped and homogenized mantle without CP were judged at the stage of initial

Table II-7. Influence of chloramphenicol (CP) on the contents of ATP and its related compounds and freshness indices in the chopped mantle during storage at 5℃.

	Storage time (days)	ATP-related compounds (μmol/g)										Freshness indices (%)			Sensory ratings*
		ATP	ADP	AMP	IMP	AdR	HxR	Hx	Ad	Xt	Total	K	K'	AEC	
Without CP	0	1.62	0.34	0.10	0.01	0	0.11	0.02	0.04	0	2.23	6	6	87	1
	1	1.15	0.40	0.20	0.07	0	0.24	0.01	0.08	0	2.16	12	16	77	1
	2	0.93	0.33	0.16	0.04	0	0.20	0.02	0.03	0	1.70	13	16	76	1
	4	0.40	0.37	0.36	0.12	0	0.15	0.01	0.02	0.01	1.43	12	20	52	1
	7	0.14	0.25	0.70	0.18	0	0.06	0.04	0.03	0.05	1.45	7	21	25	2
	10	0.12	0.05	0.56	0.16	0	0.09	0.07	0.02	0.11	1.19	15	31	20	3
With CP	0	1.62	0.34	0.10	0.01	0	0.11	0.02	0.04	0	2.23	6	6	87	1
	1	0.75	0.46	0.31	0.08	0	0.31	0.01	0.01	0	1.93	16	21	64	1
	2	0.62	0.40	0.40	0.05	0	0.26	0.01	0.01	0	1.75	16	19	58	1
	4	0.34	0.28	0.47	0.09	0	0.28	0.03	0.01	0	1.51	20	26	44	1
	7	0.26	0.30	0.52	0.25	0	0.27	0.03	0.02	0	1.64	18	34	38	1
	10	0.17	0.14	0.54	0.33	0	0.24	0.03	0.04	0	1.49	19	42	28	1

*: 1, Acceptable; 2, Initial decomposition; 3, Advanced decomposition.

Table Ⅱ-8. Influence of chloramphenicol (CP) on the contents of ATP and its related compounds and freshness indices in the homogenized mantle during storage at 5℃.

	Storage time (days)	ATP-related compounds (μmol/g)										Freshness indices (%)			Sensory ratings*
		ATP	ADP	AMP	IMP	AdR	HxR	Hx	Ad	Xt	Total	K	K'	AEC	
Without CP	0	1.62	0.34	0.10	0.01	0	0.11	0.02	0.04	0	2.23	6	6	87	1
	1	0.12	0.04	0.72	0.57	0	0.47	0.03	0.02	0.02	1.97	26	54	15	1
	2	0.10	0.03	0.28	0.83	0	0.69	0.05	0.01	0.03	2.02	37	79	29	1
	4	0.12	0.03	0.06	0.07	0	1.24	0.20	0.01	0.10	1.82	84	87	63	1
	7	0.09	0.02	0.04	0.02	0	0.24	1.02	0	0.26	1.68	89	90	68	2
	10	0.08	0.01	0.03	0.01	0	0.05	1.17	0	0.36	1.70	91	92	74	3
With CP	0	1.62	0.34	0.10	0.01	0	0.11	0.02	0.04	0	2.23	6	6	87	1
	1	0.09	0.05	0.80	0.59	0	0.40	0.03	0.02	0.01	1.99	22	52	13	1
	2	0.09	0.03	0.28	0.81	0	0.68	0.04	0.01	0.02	1.95	37	79	26	1
	4	0.11	0.02	0.06	0.42	0	1.13	0.06	0.00	0.05	1.85	66	90	63	1
	7	0.09	0.03	0.03	0.07	0	1.32	0.12	0.01	0.12	1.80	86	91	70	1
	10	0.10	0.03	0.03	0.02	0	1.33	0.17	0	0.18	1.85	89	91	72	1

*: 1, Acceptable; 2, Initial decomposition; 3, Advanced decomposition.

and advanced decomposition on the 7th and 10th day of storage, respectively. On the contrary, for portions with CP, no evidence of decomposition was detected during storage. In the chopped mantle, the changes in ATP and its related compounds proceeded much slowly than those in the adductor muscle. In the chopped mantle with CP, IMP increased continuously and HxR level was higher than those without CP during storage. Xt was detected at very low levels in the chopped mantle without CP, but not in the chopped mantle with CP. In the homogenized mantle without CP, the changes in ATP and its related compounds proceeded much faster than those in the chopped mantle (**Table II-7 and 8**). In the homogenized mantle with and without CP, ATP and ADP decreased rapidly for 1 day of storage, while AMP and IMP increased first and then decreased rapidly. In the homogenized mantle without CP, HxR increased and then decreased rapidly as the decomposition progressed. Hx and Xt also increased rapidly as the decomposition progressed. On the other hand, HxR increased continuously and the levels of Hx and Xt was low in the homogenized mantle with CP compared with those without CP. Without CP, the K value was low in the chopped mantle during storage, while the AEC value decreased continuously during acceptable stage. In the homogenized mantle, K value increased rapidly and linearly, while the AEC value fluctuated during storage.

The changes in ATP and its related compounds in the chopped and homogenized gill with and without CP during storage at 5℃ (**Table II-9 and 10**) were similar to those in the mantle (**Table II-7 and 8**). The levels of IMP, HxR, and Hx were low and Xt was not detectable during storage in the chopped gill, while those 3

Table Ⅱ-9. Influence of chloramphenicol (CP) on the contents of ATP and its related compounds and freshness indices in the chopped gill during storage at 5℃.

	Storage time (days)	ATP-related compounds (μmol/g)										Freshness indices (%)			Sensory ratings*
		ATP	ADP	AMP	IMP	AdR	HxR	Hx	Ad	Xt	Total	K	K'	AEC	
Without CP	0	1.15	0.32	0.19	0.00	0	0.11	0	0.04	0	1.80	6	6	79	1
	1	0.76	0.42	0.34	0.06	0	0.15	0.05	0.01	0	1.79	11	14	64	1
	2	0.64	0.48	0.38	0.07	0	0.15	0.04	0.02	0	1.77	11	15	59	1
	4	0.54	0.41	0.38	0.11	0	0.13	0.04	0.02	0	1.61	10	17	56	1
	7	0.27	0.26	0.50	0.16	0	0.10	0.06	0.02	0.02	1.40	12	24	39	1
	10	0.05	0.10	0.49	0.15	0	0.16	0.08	0.03	0.26	1.32	23	38	15	2
With CP	0	1.15	0.32	0.19	0.00	0	0.11	0	0.04	0	1.80	6	6	79	1
	1	0.98	0.49	0.34	0.03	0	0.15	0.02	0.01	0	2.01	8	10	68	1
	2	0.40	0.33	0.49	0.08	0	0.18	0.03	0.03	0	1.55	14	19	46	1
	4	0.20	0.25	0.47	0.15	0	0.18	0.02	0.02	0	1.29	16	28	36	1
	7	0.09	0.14	0.43	0.21	0	0.24	0.03	0.03	0	1.16	23	41	24	1
	10	0.13	0.06	0.38	0.22	0	0.25	0.06	0.03	0	1.13	29	49	28	1

*; 1, Acceptable; 2, Initial decomposition; 3, Advanced decomposition.

Table Ⅱ-10. Influence of chloramphenicol (CP) on the contents of ATP and its related compounds and freshness indices in the homogenized gill during storage at 5℃.

	Storage time (days)	ATP-related compounds (μmol/g)										Freshness indices (%)			Sensory ratings*
		ATP	ADP	AMP	IMP	AdR	HxR	Hx	Ad	Xt	Total	K	K'	AEC	
Without CP	0	1.15	0.32	0.19	0.00	0	0.11	0	0.04	0	1.80	6	6	79	1
	1	0.04	0.06	0.11	0.61	0	0.99	0.06	0.01	0.05	1.94	56	89	34	1
	2	0.05	0.07	0.04	0.08	0	1.47	0.13	0.01	0.11	1.94	87	92	53	1
	4	0.02	0.03	0.02	0.03	0	1.33	0.23	0.01	0.14	1.81	94	96	52	1
	7	0.04	0.01	0.01	0.02	0	0.75	0.81	0.01	0.24	1.90	95	96	71	2
	10	0.03	0	0.01	0	0	0.47	1.14	0	0.33	1.97	98	98	83	3
With CP	0	1.15	0.32	0.19	0.00	0	0.11	0	0.04	0	1.80	6	6	79	1
	1	0.04	0.09	0.15	0.71	0	0.96	0.08	0.01	0.03	2.06	51	86	31	1
	2	0.05	0.07	0.03	0.09	0	1.49	0.10	0.01	0.07	1.90	87	92	55	1
	4	0.04	0.05	0.02	0.01	0	1.46	0.18	0.01	0.11	1.87	93	94	63	1
	7	0.04	0.04	0.01	0.01	0	1.40	0.32	0.01	0.20	2.03	95	95	65	1
	10	0.03	0.02	0.01	0.01	0	1.36	0.38	0.00	0.25	2.05	96	97	74	1

*; 1, Acceptable; 2, Initial decomposition; 3, Advanced decomposition.

levels were high and Xt was detected in the homogenized gill.

In the chopped body trunk without CP, ATP decreased slowly, ADP and AMP accumulated at high levels, and IMP, HxR, and Hx were at low levels (**Table II-11**) similarly to those in the chopped mantle and gill (**Table II-7 and 9**, respectively), but Xt was detected even in the chopped body trunk with CP. In the homogenized body trunk both with and without CP, the breakdown of ATP to HxR proceeded very fast as shown in **Table II-12** compared with those in the other 3 homogenized tissues. Although the further breakdown of HxR to Xt in the homogenized body trunk was suppressed by the CP (**Table II-12**), the rates were very fast compared with the other 3 homogenized tissues (**Table II-6, 8, and 10**). As a result, the K and K' value in the homogenized body trunk jumped from 7-8% to about 90% for 1 day of storage.

Discussion

The author examined changes in content of ATP related compounds in various tissues of oyster when each tissue was stored as a whole tissue, and showed that the contents and degradation patterns were different among the tissues (Section II-2 and 3). In this study, when the tissue was chopped into small pieces and stored, the contents and the degradation patterns were also different among the tissues (**Table II-5, 7, 9, and 11**). However, the degradation rates of ATP and its related compounds in each chopped tissue without CP were similar to those when each tissue was stored as a whole tissue (**Figs. II-12, 13, 14, and 15** in Section II-3). In the chopped adductor muscle, a marked decrease in ATP was observed

Table II-11. Influence of chloramphenicol (CP) on the contents of ATP and its related compounds and freshness indices in the chopped body trunk during storage at 5℃.

	Storage time (days)	ATP-related compounds (μmol/g)										Freshness indices (%)			Sensory ratings*
		ATP	ADP	AMP	IMP	AdR	HxR	Hx	Ad	Xt	Total	K	K'	AEC	
Without CP	0	2.10	0.55	0.30	0.04	0	0.19	0.03	0.05	0	3.25	7	8	81	1
	1	1.94	0.62	0.31	0.06	0	0.11	0.05	0.04	0	3.13	5	7	78	1
	2	1.09	0.75	0.91	0.06	0	0.10	0.11	0.03	0	3.06	7	9	53	1
	4	0.55	0.52	1.04	0.17	0	0.14	0.05	0.05	0.00	2.52	8	14	38	1
	7	0.34	0.33	0.96	0.26	0	0.22	0.08	0.10	0.08	2.40	13	25	33	2
	10	0.25	0.19	0.53	0.11	0	0.50	0.17	0.01	0.56	2.32	38	44	35	3
With CP	0	2.10	0.55	0.30	0.04	0	0.19	0.03	0.05	0	3.25	7	8	81	1
	1	1.53	0.75	0.41	0.07	0	0.28	0.07	0.03	0	3.15	9	11	71	1
	2	1.01	0.70	0.73	0.09	0	0.17	0.06	0.00	0	2.77	8	12	56	1
	4	0.46	0.50	1.19	0.18	0	0.14	0.05	0.03	0	2.55	7	15	33	1
	7	0.44	0.41	0.96	0.31	0	0.30	0.07	0.11	0.04	2.62	15	27	36	1
	10	0.19	0.20	0.87	0.34	0	0.52	0.09	0.04	0.10	2.33	27	43	23	1

*: 1. Acceptable; 2. Initial decomposition; 3. Advanced decomposition.

Table II-12. Influence of chloramphenicol (CP) on the contents of ATP and its related compounds and freshness indices in the homogenized body trunk during storage at 5℃.

	Storage time (days)	ATP-related compounds (μmol/g)									Freshness indices (%)			Sensory ratings*
		ATP	ADP	AMP	IMP	AdR	HxR	Hx	Ad	Xt	Total	K	K'	AEC
Without CP	0	2.10	0.55	0.30	0.04	0	0.19	0.03	0.05	0	3.25	7	8	81	1
	1	0.19	0.02	0.02	0.04	0	2.07	0.58	0.01	0.31	3.23	91	92	88	1
	2	0.20	0.02	0.01	0.02	0	1.65	0.81	0.01	0.49	3.22	91	92	92	1
	4	0.15	0.02	0.01	0.01	0	1.24	1.11	0	0.53	3.07	93	93	89	1
	7	0.12	0.01	0.00	0.01	0	0.70	1.33	0	0.65	2.82	94	94	94	2
	10	0.14	0.01	0	0	0	0.42	1.60	0	0.72	2.89	93	93	97	3
With CP	0	2.10	0.55	0.30	0.04	0	0.19	0.03	0.05	0	3.25	7	8	81	1
	1	0.25	0.02	0.01	0.04	0	2.06	0.50	0.01	0.30	3.20	89	90	92	1
	2	0.19	0.02	0.01	0.01	0	1.71	0.69	0.01	0.47	3.13	92	92	93	1
	4	0.12	0.01	0.00	0.01	0	1.43	0.91	0.01	0.48	2.97	94	94	93	1
	7	0.14	0.01	0	0.00	0	1.11	0.93	0.00	0.52	2.71	93	93	96	1
	10	0.12	0.01	0	0.01	0	1.03	0.97	0	0.59	2.72	94	94	97	1

*: 1, Acceptable; 2, Initial decomposition; 3, Advanced decomposition.

together with the accumulation of AMP and IMP. In the muscle of kuruma prawn Penaeus japonicus, ATP degradation resulted in IMP accumulation, and further degradation to HxR or to Hx was very slow, but after muscle reached the stage of initial decomposition, a marked increase in Hx occurred (Matsumoto and Yamanaka, 1990). When the CP was added to the muscle, the degradation to HxR and Hx was completely inhibited. From these results, it was concluded that the degradation of ATP to IMP proceeded smoothly by the endogenous enzymes in the kuruma prawn muscle and that the increase in Hx was due to the action of bacterial enzymes (Matsumoto and Yamanaka, 1992). On the other hand, Kawashima and Yamanaka reported that both the adductor muscle of scallop Patinopecten yessoensis with and without CP added showed the same ATP breakdown pattern; the degradation to HxR to Hx was not inhibited by CP, therefore, the degradation was mainly caused by endogenous enzymes (Kawashima and Yamanaka, 1994). Although a marked increase in HxR and Hx occurred after the chopped adductor muscle of oyster without CP reached the stage of initial decomposition as in the case of kuruma prawn muscle, the degradation to HxR and Hx proceeded gradually in the chopped oyster muscle with CP (**Table II-5**) as in the case in scallop muscle. These results indicate that the degradation to HxR and Hx proceed by both the endogenous and exogenous enzymes in the chopped adductor muscle. Additionally, further degradation to Xt proceeds gradually by endogenous and exogenous enzymes from the results of Xt accumulation in the muscle with and without CP. In the case of homogenized adductor muscle of oyster (**Table II-6**), the degradation patterns of ATP and its related compounds was

generally similar to those in the chopped muscle except for the degradation of IMP to HxR, which was accelerated by the homogenization.

In the case of chopped mantle, gill, and body trunk (**Table II-7, 9, and 11**), the degradation of ATP to AMP proceeded smoothly by endogenous enzymes but the further degradation of AMP by the endogenous enzymes was very slow, differing from those in the adductor muscle of oyster (**Table II-5**) and scallop (Kawashima and Yamanaka, 1994). On the other hand, when the tissue was homogenized, the degradation of ATP to HxR by the endogenous enzymes proceeded smoothly in the mantle and gill (**Table II-8 and 10**). In the homogenized body trunk, the degradation of ATP to HxR proceeded extremely smoothly and the further degradation to Xt proceeded smoothly (**Table II-12**). **Table II-13** summarized the breakdown rates of ATP and its related compounds by the endogenous enzymes in oyster tissues and the adequate indices of the freshness. Latently, the adductor muscle of oyster has the strong activities of ATP-degradation to HxR as shown in the results of homogenized adductor muscle. However, when the three-dimensional structure of adductor muscle is maintained (whole or chopped muscle), the strong activity of IMP degradation to HxR seems to be inhibited. In other word, the homogenization, resulting in the distraction of the tissue structure, probably activates the enzyme. Although the mantle and gill latently have the strong endogenous activities of ATP-breakdown to HxR and the body trunk has the very strong activities of ATP-breakdown to HxR and strong activities of further breakdown to Xt as shown in the results of homogenized tissues (**Table II-8, 10 and 12**), when the tissue

Table II-13. ATP-breakdown by the endogenous enzymes in oyster tissues and the possible indices of freshness.

	Homogenized tissues			Chopped tissues			Index
Adductor muscle	ATP ··	HxR ·-·	Xt	ATP ·-·	IMP --	Xt	K'
Mantle	ATP ··	HxR ·-·	Xt	ATP ·-·	AMP --	Hx	AEC
Gill	ATP ··	HxR ·-·	Xt	ATP ·-·	AMP --	Hx	AEC
Body trunk	ATP —	HxR ·-·	Xt	ATP ·-·	AMP --	Xt	AEC

——, Very strong; ·-·-, Strong; ----, Weak.

structure is maintained, the enzymatic activities of ATP-breakdown are limited from ATP to AMP and further breakdown proceed very slowly in those 3 tissues as shown in the results of chopped tissues (**Table II-7, 9, and 11**). From these properties in endogenous enzymes of ATP-breakdown, the K value is considered unsuitable as the freshness index for oyster. The possible index for the freshness of oyster is the K' value of the adductor muscle or the AEC value of the mantle, gill, or body trunk.

II.5 Chemical Indices for Assessing Freshness of pelecypods during Storage

In Japan, a large amount of shellfish is consumed as fresh material for such a variety of dishes as sashimi and sushi. Retention of the freshness becomes a matter of serious concern; the chemical assessment of freshness in postmortem storage for shellfish is less established than that for fishes. Although the K value is a well-known index for measuring freshness of fish, the value was not considered suitable as a freshness index for shellfishes, such as abalone (Watanabe et al., 1992)) and scallop (Iwamoto et al.,1991; Kawashima and Yamanaka, 1992). The author evaluated the efficacy of K, K', and AEC values as for the chemical freshness index of oyster tissues (Section II-2) during storage at various temperatures (Section II-3). The K' value obtained on the adductor muscle or AEC value done on the mantle, gill, and body trunk was found to be more suitable as a freshness index for oysters because of the enzymatic property of ATP breakdown in oyster tissues (Section II-4). The usefulness of the K' and AEC values as freshness indices of other pelecypods has not been examined so far.

In this section, the author investigated the postmortem changes of level of ATP and its related compounds in the 3 species of marine pelecypod, i.e. oysters, hard clams, and ark-shells, and calculated K, K', and AEC values. The efficacy of the indices was discussed in relation to freshness.

Materials and Methods

Materials

Live cultured oysters Crassostrea gigas and hard clams Meretrix lusoria were collected from a culture farm in Matoya Bay, Mie Prefecture. Ark-shells Anadara broughtonii were purchased from a central market in Kyoto. They were artificially purified by placing them in filtered sea water sterilized by ultra-violet rays for 24 h (Satoh, 1960). After shucking and evisceration, the each adductor muscle of oyster, or each foot muscle of the ark-shell or hard clam was held in a glass vial and stored at 5°C. At each fixed time of storage, 10 specimens were used for the preparation of acid soluble fractions in the same manner as described in Section II-1.

Determination of ATP and Its Related Compounds

ATP and its related compounds, ATP, ADP, AMP, IMP, AdR, HxR, and Hx were determined by HPLC as described in Section II-1.

Calculation of Chemical Indices of Freshness

The K, K', and AEC values were calculated from the contents of ATP and its related compounds as described in Section II-2.

Organoleptic Test

The sensory ratings of the 3 species were evaluated using the organoleptic test as described in Section II-2.

Results

Changes in Content of ATP and Its Related Compounds

Figure II-20 shows the changes in average content of ATP and its related compounds in the adductor muscle of the oyster (n=10) stored at 5° C. During storage, the level of ATP decreased rapidly and reached about 0.1μmol/g after 1 day of storage. The level of ADP decreased gradually during storage. With the decrease of ATP, there was found a marked accumulation of AMP. Then with the decrease of AMP, IMP increased markedly. A small amount of AdR, which is not shown in **Fig. II-20**, was detected on the 1st (0.04μmol/g) and 3rd day (0.13μmol/g) of storage. HxR increased slowly to 1.34μmol/g until 10th day. The Hx level was very low during storage. The total content of ATP and its related

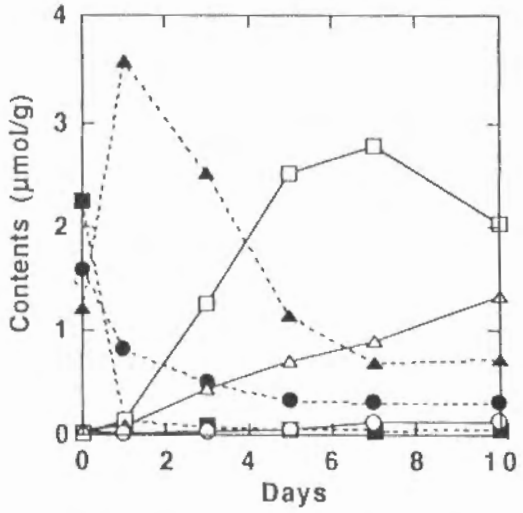

Fig. II-20 Changes in average content of ATP and its related compounds in the adductor muscle of oyster during storage at 5° C (n=10).
■, ATP; ●, ADP; ▲, AMP;
□, IMP; △, HxR; ○, Hx.

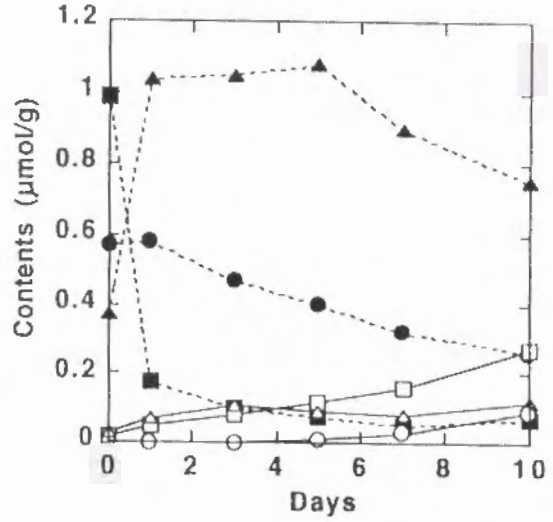

Fig. II-21 Changes in average content of ATP and its related compounds in the foot muscle of hard clam during storage at 5° C (n=10). Symbols are the same as those given in the footnote of **Fig. II-20**.

compounds, about 5μmol/g, was relatively constant during storage.

In the foot muscle of the hard clam, the level of ATP decreased rapidly and reached 0.18μmol/g after 1 day of storage, and thereafter decreased slowly (**Fig. II-21**). The level of ADP was constant for 1 day of storage, and then it decreased gradually. With the rapid decrease of ATP within 1 day of storage, AMP accumulated markedly, then remaining constant for 5 days, and thereafter decreased. The rate of increase in the IMP level in the hard clam muscle was very low compared with that in the oyster muscle. The levels of HxR and Hx were low; AdR was undetectable. The total content of ATP and its related compounds in the hard clam muscle, about 1.9μmol/g, was much lower than that in the oyster muscle.

In the foot muscle of the ark-shell, the level of ATP decreased linearly from 3.12 to 0.10μmol/g during the 7 days of storage (**Fig. II-22**). The rate of decrease in the ATP level in the ark-shell muscle was very low compared with those in the oyster and hard clam muscle. The level of ADP increased to 1.50μmol/g on the 1st day, and then it decreased. The level of AMP increased linearly and rapidly until 3rd day, increased slowly until 7th day, and then decreased. The level of IMP increased linearly from 0.01 to 2.10μmol/g until 10th day. A small amount of AdR was also detected (data not shown). The levels of HxR and Hx remained low. The total content of ATP and its related compounds was about 4.6μmol/g in the ark-shell muscle.

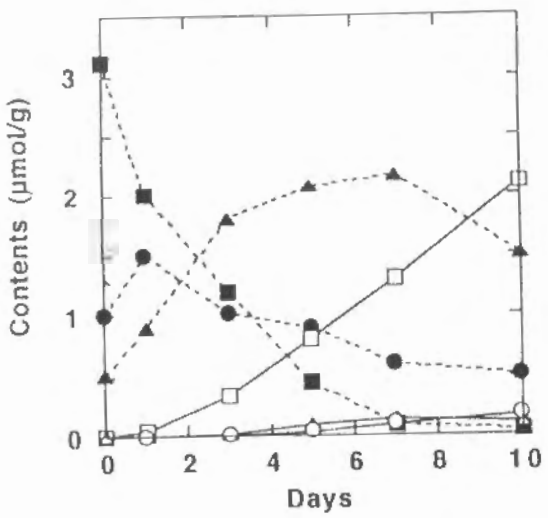

Fig. II-22 Changes in average content of ATP and its related compounds in the foot muscle of ark-shell during storage at 5°C (n=10). Symbols are the same as those given in the footnote of **Fig. II-20.**

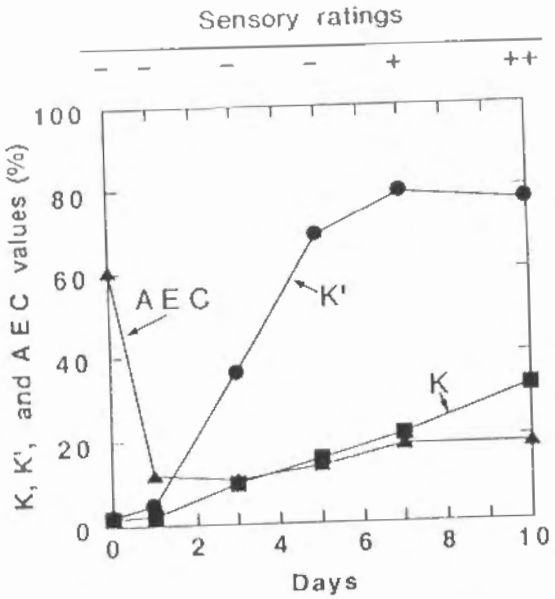

Fig. II-23 Changes in average K (■), K' (●), and AEC (▲) values in the adductor muscle of oyster during storage at 5°C (n=10) together with sensory ratings. -, acceptable; +, initial decomposition; ++, advanced decomposition.

Chemical Freshness Indices

Figures II-23, 24 and 25 show the changes in K, K', and AEC values with sensory ratings obtained on the muscle of oyster, hard clam, and ark-shell, respectively. On the 7th day of storage of the oyster, hard clam, and ark-shell, the muscle gave off a faintly putrid smell and this stage was recognized as the stage of initial decomposition. On the oyster muscle, the K value increased linearly but slowly, and reached to the level of 20% at the initial decomposition stage (**Fig. II-23**). The K' value increased linearly and rapidly during an acceptable stage, and reached the level of about 80% at the initial decomposition stage, thereafter it remained constant. The AEC value fell from 60% to 12% during 1 day of storage. On the hard clam muscle, the K value was at low levels

during acceptable stage, thereafter it increased slowly (**Fig. II-24**). The K' value increased slowly but linearly as the changes in K value obtained on the oyster muscle. The AEC value obtained on the hard clam muscle dropped from 66% to 26% during 1 day of storage, thereafter it decreased gradually. On the ark-shell muscle, the K value increased very slowly and the value was only 6% at the initial decomposition stage (**Fig. II-25**); the K' value increased rapidly and linearly from the beginning of storage. The AEC value also changed rapidly and linearly from the beginning of storage.

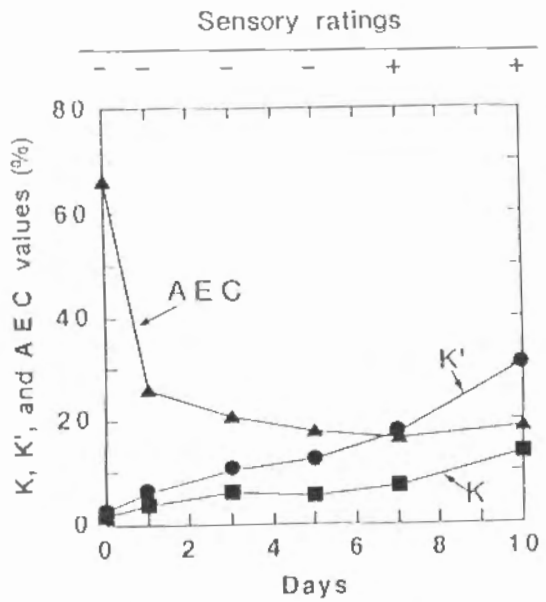

Fig. II-24 Changes in average K, K', and AEC values in the foot muscle of hard clam during storage at 5°C (n=10) together with sensory ratings. Symbols are the same as those given in the footnote of **Fig. II-23.**

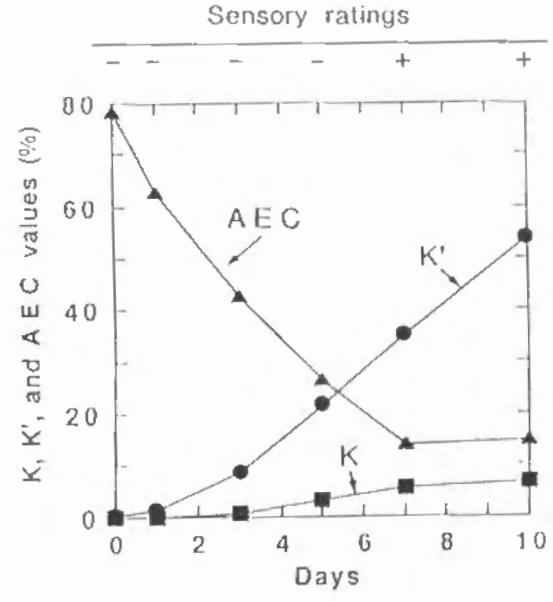

Fig. II-25 Changes in average K, K', and AEC values in the foot muscle of ark-shell during storage at 5°C (n=10) together with sensory ratings. Symbols are the same as those given in the footnote of **Fig. II-23.**

Discussion

The average AEC value at time 0 obtained on the muscle of the oyster, hard clam, and ark-shell was 60, 66, and 78%, respectively (**Figs. II-23, 24, and 25**), and was much higher than that obtained on the muscle of the oyster, hard clam, and ark-shell in the report by Suwetja et al. (1989) (about 40, 23, and 39%, respectively, which were calculated from the contents of ATP, ADP, and AMP in their report). The energy charge of the adenylate systems has been proposed by Atkinson (1968) as a fundamental control parameter of metabolism. This concept implies that the conservation of ATP is a major feature of metabolic regulation. The high levels of AEC and ATP in the muscle of the oyster, hard clam, and ark-shell obtained in this investigation indicated that specimens used in this study were under a better condition before the experiment than those in the report by Suwetja et al. (1968). In fact, in the preliminary experiment, the author confirmed that the AEC value at time 0 decreased when the specimens were exposed to the air for few days. The degradation patterns of ATP and its related compounds differed among the 3 species. ATP decreased rapidly in the muscle of oyster (**Fig. II-20**) and hard clam (**Fig. II-21**), but gradually in the muscle of ark-shell (**Fig. II-22**) as did those in the mantle, gill, and body trunk of oyster in Section II-2 and 3. AMP accumulated in the muscle of all 3 species, while further degradation to IMP and/or HxR occurred markedly in the muscle of oyster and ark-shell. These findings suggested that the activities of the enzyme systems responsible for the degradation of ATP, ADP, AMP, and IMP, that is ATPase, myokinase, AMP deaminase, and 5'-

nucleotidase, respectively, differed among the 3 species. Both IMP and AdR were detected in the muscle of the oyster and ark-shell. The author showed previously the two pathways of AMP degradation in the oyster muscle in Section II-2. In this study, the author also found two pathways of AMP degradation, IMP pathway and AdR pathway, in the foot muscle of the ark-shell during storage. In the foot muscle of the hard clam, a small amount of IMP was detected, but AdR was undetectable, suggesting the IMP pathway functions predominantly during storage.

As the chemical freshness indices of the oyster, hard clam, and ark-shell, K, K', and A.E.C. values were calculated from the levels of ATP and its related compounds (**Figs. II-23, 24, and 25**). The author previously showed that the K' value in the adductor muscle or the AEC value in the mantle, gill, and body trunk that showed a rapid and linear change from the beginning of storage might be useful as a freshness indicator for the oyster in Section II-2, 3, and 4. In this study, the author confirmed the findings that the K' value in the oyster muscle was useful as a freshness indicator for the oyster because of its linear and rapid increase during the acceptable stage (**Fig. II-23**). In the foot muscle of the hard clam, the K value showed little increase during the acceptable stage and the AEC value did not change linearly (**Fig. II-24**). The K' value increased slowly from the beginning of storage to about 20% by the initial decomposition stage. However, the K' value in the muscle of hard clam was useful as a freshness indicator because of its linear increase during the acceptable stage as suggested by Sakaguchi et al. (1990) on the K value on the muscle of oyster C. gigas and by Matsumoto and Yamanaka (1990) on

the K value on the muscle of prawn Penaleus japonicus. In the ark-shell, the AEC and K' values seemed to be very useful as freshness indices because of their linear and rapid changes from the beginning of storage (**Fig. II-25**). In conclusion, the K value could not be used as a freshness index for the hard clam or ark-shell. The K' value and AEC value were potential indices for the freshness of pelecypods such as oyster, hard clam, and ark-shell, and thus an adequate index for respective species of pelecypod could be used. However, these results on the freshness indices calculated from the contents of ATP and its related compounds were obtained from investigations using muscles stored at 5° C. Although the author showed that the K' and AEC values were useful freshness indices for oyster tissues at various storage temperature, an interesting effects of storage temperature on the ATP degradation, a faster ATP degradation at a lower storage temperature, has been observed in the muscle of other shellfishes such as disk abalone (Watanabe et al., 1992) and scallop (Iwamoto et al., 1991; Kawashima and Yamanaka, 1992). In order to determine whether or not the K' and AEC values can be applied to pelecypods as freshness indices at other temperatures of storage, further study is needed.

II.6 Phosphorus-31 nuclear magnetic resonance study of postmortem changes in oyster tissues

In vivo phosphorus-31 nuclear magnetic resonance (^{31}P NMR) has been used so far for measuring serial changes in high energy phosphate metabolism (Gadian, 1982). Concentrations of high energy phosphate metabolites, intracellular pH and Mg^{2+} ion concentrations can be measured, which allows to evaluate the fatigue status of the muscle (Gupta and Gupta, 1987). In Section II-2 and 3, the author showed that the change in patterns and rates of ATP degradation during storage were much different among oyster tissues. The author also showed that the PCr acts as the energy reservoir for maintaining the ATP levels even in the muscle excised from fish as observed in the case of living fish muscle (Van den Thillart et al., 1989). One of the possible reasons for the differences of ATP breakdown among the oyster tissues is the differences of PAr levels among those tissues. It is interesting whether or not the level of PAr, which is a well-known energy-reservoir of ATP in mollusks, is different among the oyster tissues.

In Section II-2, 3, and 4, the author showed the possible chemical indices for the freshness of oyster using HPLC. Although they were effective indicators for the freshness of oyster, it was troublesome for the measurement to homogenize the tissue, extract and separate the compounds each other by chromatography.
In Section I-2, the author showed that ^{31}P NMR is a possible method for evaluating fish meat quality, such as freshness, because of its non-invasive, convenient, simultaneous, and rapid

determination of high energy phosphate compounds in the tissue of fish.

In this section, the author examined the change in levels of high energy phosphate compounds, pH, and Pi using ^{31}P NMR during storage of the oyster tissues. The author also discussed the efficacy of ^{31}P NMR to estimate the freshness of oyster.

Materials and Methods

Materials

Live cultured oyster Crassostrea gigas was collected from a cultured farm in Matoya Bay, Mie Prefecture. They were artificially purified (Satoh, 1960) as described in Section II-2. Whole oyster flesh was removed by cutting the adductor muscle close to the valves and held in a plastic bag.

^{31}P NMR Measurement of Carp Muscle

NMR measurements were made with a JNM GSX-270 spectrometer system (JEOL, Japan). The surface coil (11 mm in diameter, five turn) for NMR detection was placed against the center of adductor muscle or body trunk (stomach and digestive diverticula). The spectra were obtained at an operating frequency of 109.14 MHz for phosphorus. Radiofrequency pulses of 20 μsec duration were delivered every 6.0 sec for NMR acquisition. It took 20 min to accumulate 200 scans for each measurement. The oyster flesh was fixed on the NMR probe for 25 h, and spectra were obtained at 0, 2, 4, 6, 10, 15, 20, and 25 h. The criterion for chemical shift used was 0 ppm PAr. Changes in concentration of each compounds were

determined from the area of resonance line of each signal. In the adductor muscle of oyster, relative index of concentration of [Pi], [PAr], [ATP (β-ATP)], [ATP + ADP (γ-ATP)] was obtained by the ratios of [PAr]/[Pi], [ATP (β-ATP)]/[Pi], and [ATP + ADP (γ-ATP)]/[PI]. In the body trunk of oyster, relative index of concentration [PME + Pi + PDE], [PAr], [ATP (β-ATP)], [ATP + ADP (γ-ATP)] was obtained by the ratios of [PAr]/[PME + PI + PDE], [ATP (β-ATP)]/[PME + Pi + PDE], and [ATP + ADP (γ-ATP)]/[PME + Pi + PDE], because the resonance lines of PME, Pi, and PDE were overlapped and became difficult to distinguish each other at 10 h or later as shown in **Fig. II-26**.

Determination of Intracellular pH

The intracellular pH (pHi) in oyster tissue was estimated on the basis of the differences between the chemical shifts of Pi and of PAr by constructing a pH titration curve with a standard solution (H_3PO_4, 50 mM; PCr, 25 mM; KCl, 100 mM; and $MgCl_2$, 1 mM). The chemical shift of PAr was postulated as 0 ppm. From the chemical shift (δppm) of the Pi resonance pHi was calculated by use of the following equation (Gadian et al., 1979; Seo et al., 1983).

$$pHi = pK' + \log (\delta - \delta_\alpha) / (\delta_\beta - \delta)$$

where $pK' = 6.87$, $\delta_\alpha = -3.17$ ppm, $\delta_\beta = -5.61$ ppm were used.

Results and Discussion

Postmortem changes of typical ^{31}P NMR spectra of the adductor muscle and body trunk of oyster obtained at time 0 are shown in **Fig. II-26 (A) and (B)**, respectively. In both tissues, there were

seven main peaks, assigned, from left to right, to PME, Pi, PDE, PAr, γ-ATP(γ-phosphate of ATP + β-phosphate of ADP), α-ATP [α-phosphate of ATP + α-phosphate of ADP + NAD(H)], and β-ATP (β-phosphate of ATP). These peak assignments were performed according to Barany and Glonek (1982). In the adductor muscle, the Pi level was high and PAr level was low compared with those in the body trunk. The stress deceased the PCr level and increased the Pi level in the carp muscle immediately after death as shown in Section I-2. Chiba et al., (1991) reported similar results that the PCr level decreased and Pi level increased due to muscle fatigue caused by capture. The stress of cutting muscle close to

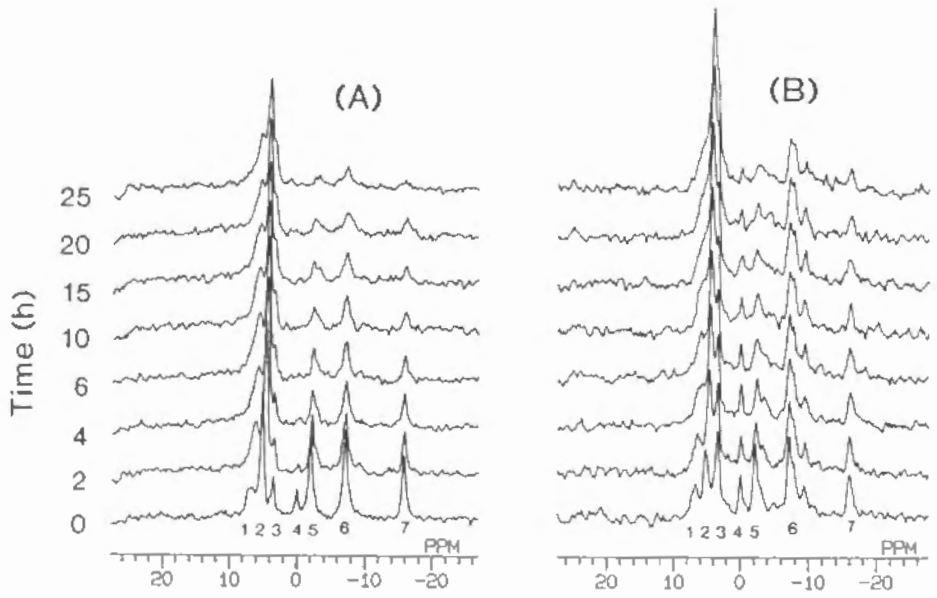

Fig. II-26 Changes of ^{31}P NMR spectra of the adductor muscle (A) and body trunk (B) of oyster during storage at 22-23° C. Peak assignments are as follows: 1, PME (SP + IMP); 2, Pi; 3, PDE; 4, PAr; 5, γ-ATP (γ-phosphate of ATP + β-phosphate of ADP); 6, α-ATP [α-phosphate of ATP + α-phosphate of ADP + NAD(H)], and 7, β-ATP (β-phosphate of ATP).

the valves could be one of the reasons of the differences of PAr and Pi levels between the oyster tissues. During storage, the levels of PAr, α-, β-, and γ-ATP decreased and the Pi levels increased in both tissues.

PDE (mainly glycerophosphorylcholine (GPC) judging from the chemical shift) was detected both in the adductor muscle and body trunk of oyster. This compound has been reported to occur in some rabbit (Burt et al., 1976; Renou et al., 1986; Seeley et al., 1976), porcine (Uhrin and Liptaj, 1991), frog and toad (Burt et al., 1976), rat and goat (Azuma et al., 1994a and b) skeletal muscles, and in tortoise, rabbit and bovine heart muscles (Burt et al., 1976), as well as in human leg muscles of patients with some neuromuscular diseases (Barany et al., 1989). Although it is generally considered that GPC is related to the metabolism of red or slow-twitch myofibers, the physiological and biochemical roles of this metabolite are not well understood. Schmidt et al., (1952) reported that GPC may be a cell membrane breakdown product arising from phospholipids. This may partially explain the higher PDE (GPC) level in the body trunk than that in the adductor muscle of oyster, since the body trunk included digestive diverticula. At present, the author cannot explain, however, the role of PDE in oyster tissues.

Figure II-27 showed the changes in the levels of β-ATP, which means [ATP], and γ-ATP, which means [ATP + ADP] compared with the initial levels. β-ATP of the adductor muscle decreased rapidly to 48±11% of at 2 h postmortem, and thereafter decreased gradually. β-ATP of the body trunk decreased gradually during 10 h storage, and thereafter it remained at about 50% levels. γ-ATP also

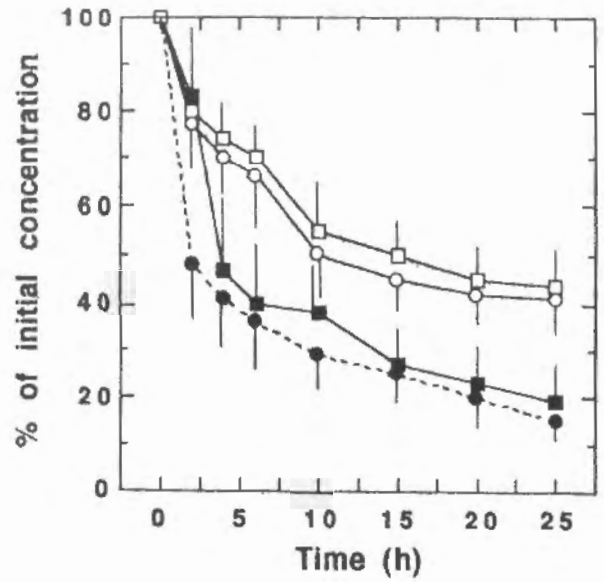

Fig. II-27 Changes in β-ATP (β-phosphate of ATP) and γ-ATP (γ-phosphate of ATP + β-phosphate of ADP) levels expressed as a percentage of the initial value of adductor muscle and body trunk of oyster. ●, β-ATP of adductor muscle; ■, γ-ATP of adductor muscle; ○, β-ATP of body trunk; □, γ-ATP of body trunk. Values are the mean±S.D. (n=5).

decreased slowly in the body trunk compared with those in the adductor muscle. Previously, using HPLC, the author showed that the ATP and ADP contents decreased faster in the adductor muscle than in the other tissues of oyster in Section II-2 and 3.
The results on the ATP and ADP changes of oyster tissues by ^{31}P NMR agree with these previous findings. The changes in levels of PAr and Pi were also different between the adductor muscle and the body trunk (**Fig. II-28**). The PAr level in the adductor muscle decreased fast and disappeared by 4 h. On the contrary, the PAr level in the body trunk decreased gradually and still remained about 50% of initial level at 25 h. These differences in the levels of PAr, as an energy reservoir, seem to explain the earlier decrease of ATP in the adductor muscle than in the body trunk. The

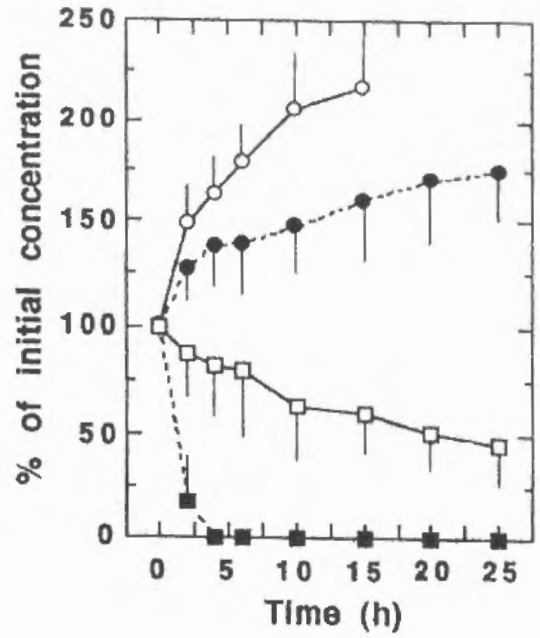

Fig. II-28 Changes in PAr and Pi levels expressed as a percentage of the initial value of adductor muscle and body trunk of oyster. ■, PAr of adductor muscle; ●, Pi of adductor muscle; □, PAr of body trunk; ○, Pi of body trunk. Values are the mean±S.D. (n=5).

Pi level in the body trunk increased faster than in the adductor muscle, since the initial Pi content in the body trunk was much low as shown in the **Fig. II-26**. (The Pi level of body trunk at 20 and 25 h did not show, since the separation and estimation of the peak areas of Pi, PME, and PDE became difficult at 10 and 15 h and thereafter it became impossible as shown in the **Fig. II-26**.)

It is one of the most attractive features of NMR that intracellular pH can be measured non-invasively with no need for a micro electrode to be placed into the cell or the use of chemicals that affect cellular conditions. The time course of pHi decline was obtained by repeated measurements in a single specimen. At time 0, the pHi in the adductor muscle and the body trunk were 7.33±0.07 and 7.48±0.06, respectively (**Fig. II-29**).

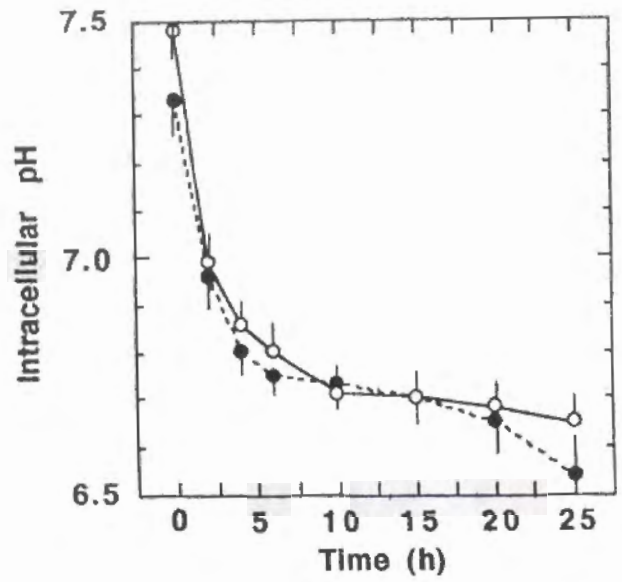

Fig. II-29 Time courses of pHi changes in the adductor muscle (●) and the body trunk (○) of oyster. Values are the mean±S.D. (n=5).

There was statistically significant difference between the values ($p<0.05$, Student's t-test). In the adductor muscle and body trunk, pHi decreased rapidly to 6.97±0.05 and 6.99±0.06, respectively at 2 h. The changing rates and patterns of pHi in the adductor muscle and the body trunk were similar each other from 2 h to 20 h storage and there were not statistically significant differences between the values in the two tissues. Although pHi in the adductor muscle tended to decrease from 20 h to 25 h storage, there was not significant differences between the values at 25 h. It was interesting that pHi in the body trunk became constant around 6.7 from 10 h to 25 h storage. The ultimate pH in postmortem skeletal muscles is, in general, approximately 5.5 (Greaser, 1986). In our preliminary experiments with a longer observation period (48 h), there was small decline of pHi in the adductor muscle to around 6.5

and in the body trunk around 6.6, indicating the ultimate pH depends on the animal species.

Figure II-30 and 31 show the time courses of mean [PAr]/[Pi (+ PME + PDE)], [β-ATP]/[Pi (+ PME +PDE)], and [γ-ATP (ATP + ADP)]/[Pi (+ PME +PDE)] ratios in the adductor muscle and body trunk of oyster, respectively. The [PAr]/[Pi] ratio of adductor muscle of oyster decreased rapidly from 0.112±0.014 to 0.01±0.006 at 0 and 2 h storage, respectively. The [β-ATP]/[Pi] and [γ-ATP]/[Pi] ratios in the adductor muscle decreased rapidly during 4 h storage, thereafter slowly to 10 h. On the other hand, the three ratios in the body trunk decreased similarly and continuously during 10 h storage. The specimen of whole oyster flesh kept in a plastic bag at 23° C, the same temperature of NMR measurement,

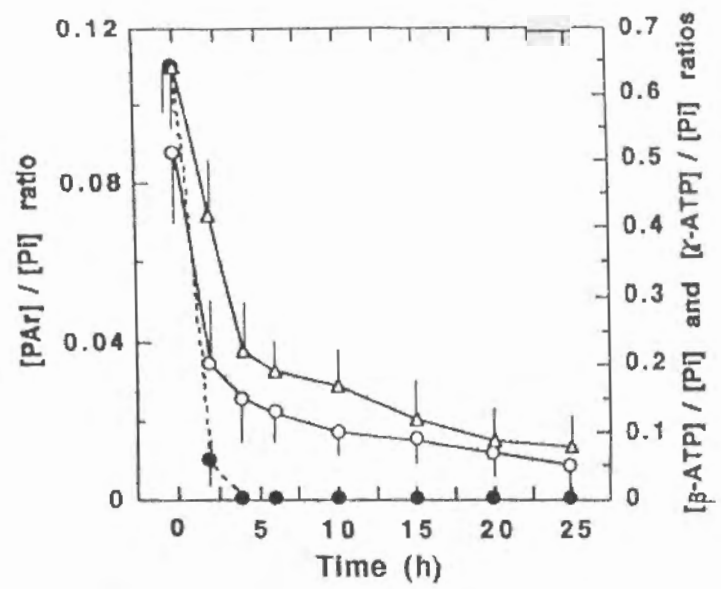

Fig. II-30 Time courses of [PAr]/[Pi] (●), [β-ATP (ATP)]/[Pi] (○), and [γ-ATP (ATP + ADP)]/[Pi] (△) ratios in the adductor muscle of oyster. Values are the mean±S.D. (n=5).

emitted a faintly putrid smell at 8-10 h storage and the stage was recognized as the initial decomposition. For the evaluation of meat quality, such as freshness, the index must give information before the decomposition stage. In other words, the changing magnitudes of freshness index should be large during acceptable stage. The decrease of [PAr]/[Pi] and [β- or γ-ATP]/[Pi] in adductor muscle were much rapid during 2 to 4 h storage. They seem to be useful as the freshness indices in the earlier period of storage of oyster. The three ratios in the body trunk decreased rapidly and continuously during 8 h storage (**Fig. II-31**) and the magnitude of change was large compared with the K value of body trunk in Section II-2 and 3 (**Fig. II-11 and 19**). The three ratios

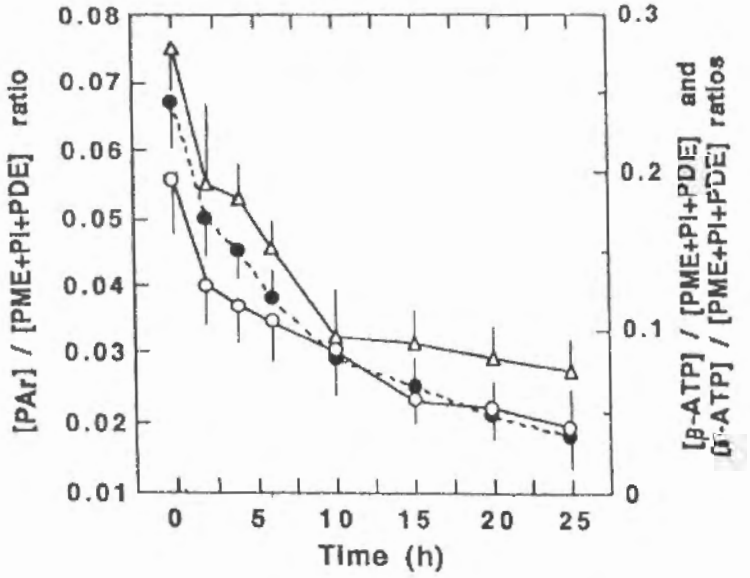

Fig. II-31 Time courses of [PAr]/[PME + Pi + PDE] (●), [β-ATP (ATP)]/[PME + Pi + PDE] (○), and [γ-ATP (ATP +ADP)]/[PME + Pi + PDE] (△) ratios in the body trunk of oyster. Values are the mean±S.D. (n=5).

of body trunk seem to be suitable index for the evaluation of freshness in oyster. Additionally, pHi in both the adductor muscle and body trunk decreased rapidly during 10 h postmortem (**Fig. II-29**). Judging from the several parameters obtained simultaneously by ^{31}P NMR, such as the ratio of high energy phosphates to Pi (+ PME + PDE) and pHi, it will possible to evaluate oyster quality as described previously on quality evaluation of carp muscle in Section I-2. These results suggested that the ^{31}P NMR is a possible tool for the evaluation of oyster freshness, because of its non-invasive, convenient, rapid, and simultaneous determination of high energy phosphate compounds, Pi, and pHi in the tissue of oyster.

Chapter III. Postmortem changes of high energy phosphate compounds and freshness indices in gastropods

The utilization and consumption of marine invertebrates as fresh material for a variety of dishes is very high in Japan, compared with other countries. There have been many reports on the postmortem changes of ATP and its related compounds and freshness in the muscle of fish, while little information has been available for the muscle of mollusks, especially for gastropod. Arai (1961) investigated the changes in the content of acid soluble nucleotides in the muscle of Yezo abalone Haliotis discus hannai stored at -5 and 20° C and reported that ATP decomposed along with ADP and AMP accumulation, while IMP, HxR, and Hx were undetectable. The freshness index has not been established for gastropods. The author evaluated the efficacy of K, K', and AEC values as the chemical freshness index, using oyster tissues (Section II-2), during storage at various temperatures (Section II-3). The K' value obtained on the adductor muscle or AEC value done on the mantle, gill, and body trunk was shown to be more suitable as a freshness index for oysters because of the enzymatic property of ATP breakdown in oyster tissues (Section II-4). The usefulness of the K' and AEC values as freshness indices of other pelecypods was also shown in Section II-5.

In this chapter, the author therefore investigated the postmortem changes in levels of ATP and its related compounds in the abalone Haliotis discus, marine gastropod, and calculated the 3 indices, K, K', and AEC values, in relation to freshness.

Materials and Methods

Materials

Abalones Haliotis discus (14.4±1.2 cm in shell length, 298±35 g in body weigh, n=10) were purchased from a central market in Kyoto. They were artificially purified by placing them in filtered sea water sterilized by ultra-violet rays for 24 h (Satoh, 1960). After shucking and evisceration, the each foot muscle of the abalone was held in a plastic bag and stored at 5° C. At each fixed time of storage, a 1 g-portion of muscle was newly cut from each muscle piece and used for the preparation of acid soluble fractions in the same manner as described in Section II-1.

Determination of ATP and Its Related Compounds

ATP and its related compounds, ATP, ADP, AMP, IMP, AdR, HxR, and Hx were determined by HPLC as described Section II-1.

Calculation of Chemical Indices of Freshness

The K, K', and AEC values were calculated from the contents of ATP and its related compounds as described in Section II-2.

Organoleptic Test

The sensory ratings of the 4 species were evaluated using the organoleptic test as described in Section II-2.

Results

Changes in Content of ATP and Its Related Compounds

Figure III-1 shows the changes in average content of ATP and its

related compounds in the foot muscle of abalone (n=10) stored at 5°C. The level of ATP decreased linearly from 2.50 to 0.46 μmol/g during the 5 days of storage, thereafter decreased gradually. The rate of decrease in ATP level in the abalone muscle was similar to that in the ark-shell muscle (**Fig. II-22** in Section II-5), and was very low compared with those in the oyster and hard clam muscle (**Fig. II-20 and 21** in Section II-5). The level of ADP increased to 1.27 μmol/g on the 2nd day, and then it decreased. The level of AMP increased linearly during storage from 0.50 to 2.10 μmol/g. The level of IMP was low, being about 0.05-0.15 μmol/g. A small amount of AdR was also detected as found in the oyster and ark-shell muscle. The level of HxR increased very slowly from 0.01 to 0.23 μmol/g during storage. The level of Hx was constant at a very low level up to 7th day, then it increased to 0.26 μmol/g on the 10th day. The total content of ATP and its related compounds was about 3.9 μmol/g in the abalone muscle.

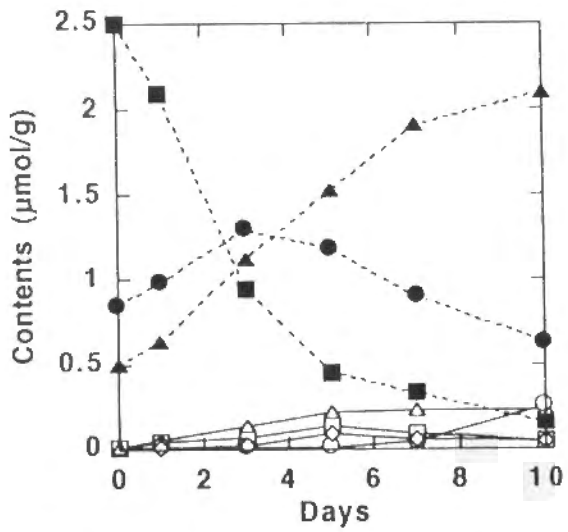

Fig. III-1 Changes in average content of ATP and its related compounds in the foot muscle of abalone during storage at 5°C (n=10).
■, ATP; ●, ADP; ▲, AMP; □, IMP; ◇, AdR; △, HxR; ○, Hx.

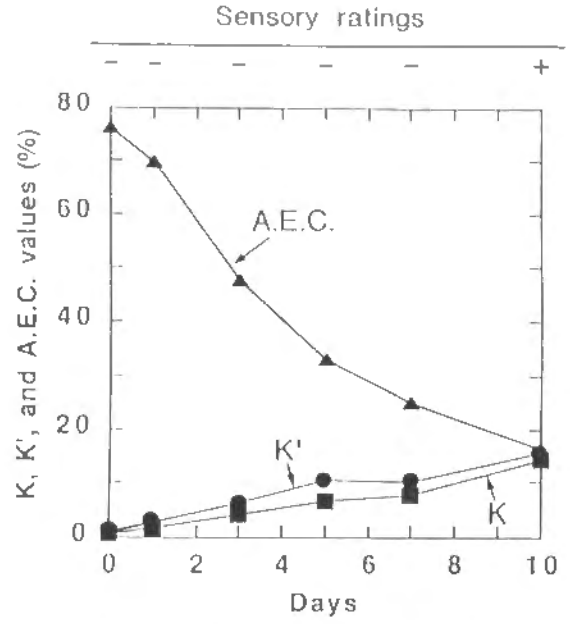

Fig. III-2 Changes in average K (■), K' (●), and AEC (▲) values in the foot muscle of abalone during storage at 5°C (n=10) together with sensory ratings.
–, acceptable; +, initial decomposition; ++, advanced decomposition.

Chemical Freshness Indices

Figure III-2 shows the changes in K, K', and AEC values with sensory ratings obtained on the muscle abalone. On the 10th day of storage of the abalone, the muscle gave off a faintly putrid smell and this stage was recognized as the stage of initial decomposition. In the abalone muscle, the AEC value decreased rapidly and linearly during storage from 76% to 17% at time 0 and on the 10th day of storage, respectively, while the K and K' value were at low levels in the acceptable stage.

Discussion

The AEC value of the abalone muscle at time 0 was high, approximately 76%, and was the same level as that reported by

Watanabe et al. (1992) using the muscle of abalone which was treated within 20 min after collection from the sea. The energy charge of the adenylate systems proposed by Atkinson (1968) as a fundamental control parameter of metabolism implies that the conservation of ATP is a major feature of metabolic regulation. The high levels of AEC and ATP in the muscle of the abalone obtained in this investigation indicated that specimens used in this study were under a better condition before the experiment.

The degradation patterns of ATP and its related compounds in abalone muscle differed from those in pelecypods, such as oyster, hard clam, and ark-shell. ATP decreased gradually in the abalone muscle (**Fig. III-1**) as did those in the mantle, gill, and body trunk of oyster (Section II-2 and 3) and that in the ark-shell muscle, while rapidly in the muscle of oyster and hard clam as shown in Section II-5. AMP accumulated in the abalone muscle as did those in pelecypods tissues, while further degradation to IMP and/or HxR did not occur markedly in the abalone muscle as those in the hard clam muscle. These findings suggested that the activities of the enzyme systems responsible for the degradation of ATP, ADP, AMP, and IMP, that is ATPase, myokinase, AMP deaminase, and 5'-nucleotidase, respectively, differed among the species. Both IMP and AdR were detected in the abalone muscle. The author previously showed the two pathways of AMP degradation in the muscle of oyster and ark-shell in Section II-2, 3, and 5. In this study, the author also found two pathways of AMP degradation, IMP pathway and AdR pathway, in the foot muscle of the abalone during storage.

As the chemical freshness indices of the abalone, K, K', and AEC

values were calculated from the levels of ATP and its related compounds (**Fig. III-2**). The author previously described that the K' value in the adductor muscle of oyster and the foot muscle of ark-shell and hard clam, or the AEC value in the other tissues of oyster and the foot muscle of ark-shell might be useful as a freshness indicator for the oyster, hard clam, or ark-shell because of its linear and rapid increase during the acceptable stage (Section II-2, 3, and 5). Watanabe et al. (1992) reported that the K value on the muscle of abalone was not suitable as a freshness index, since the value did not change from the beginning of storage and the rate of increase was slow. In this study, the K value on the abalone muscle also increased very slowly (**Fig. III-2**) and was not considered to be suitable as a freshness index. Although the K' value also increased very slowly, the AEC value changed rapidly and linearly from the beginning of storage and seemed to be very useful as a freshness index for abalone. In conclusion, the K value could not be used as a freshness index for the abalone. The AEC value was potential index for the freshness of abalone. However, these results on the freshness indices calculated from the contents of ATP and its related compounds were obtained from investigations using muscles stored at 5°C. An interesting effects of storage temperature on the ATP degradation, a faster ATP degradation at a lower storage temperature, has been observed in the muscle of some shellfishes (Watanabe et al., 1992; Iwamoto et al., 1991; Kawashima and Yamanaka, 1992). In order to determine whether or not the AEC values can be applied to abalone as freshness index at various temperatures, further study is needed.

Chapter IV. Postmortem changes of high-energy phosphate compounds and freshness indices in cephalopods

The degradation pathway of ATP in the squid muscle have been reported to proceed as ATP → ADP → AMP → AdR → HxR → Hx by Saito et al. (1958), differing from that in fishes as reviewed by Saito (1961), and Uchiyama and Ehira (1970). Arai (1966) using the muscles of common squid Todarodes pacificus and spear squid Doryteuthis bleekeri, Iwamoto and Uchiyama (1969) using the mantle muscle of "kensaki ika" D. kensaki, and Ohashi et al.(1991) using the mantle muscle of common squid also reported that IMP was undetectable and AMP degraded through the AdR pathway during storage; they failed to detect AdR in the muscle tissue. Nakamura et al. (1985), on the other hand, detected a small amount of IMP in the mantle muscle of common squid during storage. Suwetja et al. (1989) also detected IMP but not AdR in the muscle of "jindo ika" Loligo japonica. These findings suggested the occurrence of IMP pathway in AMP degradation. In those reports, ATP and its related compounds were analyzed only in the mantle or unspecified muscle of each species. The postmortem change of ATP and its related compounds in the arm or fin muscle remains unclear.

Although the K value (Saito et al., 1959) calculated from the contents of ATP and its related compounds is a well-known index for assessing the freshness of fish, it has been reported unsuitable as a freshness index for common squid (Ohashi et al., 1991; Nakamura et al., 1985). Ohashi et al.(1991) suggested that the Hx/AMP ratio was a better index of freshness of common squid

than the K value. The author previously showed the efficacy of K' and AEC values as chemical freshness indices during storage of several species of shellfishes in Chapter II and III. The usefulness of the K' value, AEC value, and Hx/AMP ratio as freshness indices of the spear squid has not been described so far.

Herein, the author investigated the postmortem changes in level of ATP and its related compounds in the mantle, arm, and fin muscles of spear squid, commercially one of the most important cephalopod as fresh material such popular dishes as sashimi and sushi, and calculated the 6 indices, K, K', and A.E.C. values and Hx, Xt, and Hx/AMP ratios in relation to freshness.

Materials and Methods

Materials

The experiments were carried out on five numbers of live spear squids Doryteuthis bleekeri which were purchased from a retailer of live fish in Kyoto. The average sample weight of squid was 385±21g (mean±S.D., n=5). Each squid was eviscerated and dissected into 3 tissues, mantle, arm, and fin muscles. Each specimen of 3 tissues was held separately in a plastic bag and stored at 5° C. At each sampling, a 1g-portion of muscle was newly cut from the muscle piece and submitted for the preparation of acid soluble fractions in the same manner as described in Section II-1.

Determination of ATP and Its Related Compounds

ATP and its related compounds, i.e. ATP, ADP, AMP, IMP, AdR,

HxR, Hx, and Xt, were determined by HPLC in the same manner as descried in Section II-1.

Calculation of Chemical Indices of Freshness

The K (Saito et al., 1959), K', and AEC (Atkinson, 1968) values were calculated from the contents of ATP and its related compounds in the same manner as described in Section II-2. The Hx and Xt ratios were calculated from the following equation:

Hx ratio (%) = (Hx) / (ATP + ADP + AMP + IMP + AdR + HxR + Hx) x 100

Xt ratio (‰) = (Xt) / (ATP + ADP + AMP + IMP + AdR + HxR + Hx + Xt) x 1000

The ratio of Hx/AMP (Ohashi et al., 1991) was also calculated from the contents of AMP and Hx as a freshness index.

Organoleptic Test

The sensory ratings of the 3 muscle tissues were evaluated using the organoleptic test (Matsumoto and Yamanaka, 1990) in the same manner as described in Section II-2.

Results

Changes in Content of ATP and Its Related Compounds

Figure IV-1 shows the changes in average content of ATP and its related compounds in the mantle muscle of spear squid (n=5) stored at 5°C. During storage, ATP level decreased rapidly from 6.5±0.3 (mean±S.D.) at time 0 to 2.9±0.8 and 0.2±0.1 μmol/g after 4 h and 1 day of storage, respectively. ADP level was constant during 4 h of storage. Then it decreased to 0.5±0.2 μmol/g after 1 day of storage, and thereafter remained constant. With the decrease of

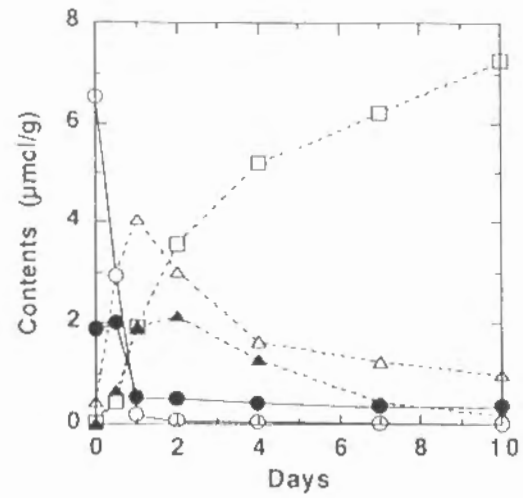

Fig. IV-1 Changes in average content of ATP and its related compounds in the mantle muscle of spear squid during storage at 5°C (n=5).
○, ATP; ●, ADP; △, AMP; ▲, HxR; □, Hx.

ATP and ADP, there was a marked accumulation of AMP and HxR. Then with the decrease of AMP and HxR, Hx increased markedly. A small amount of IMP and also AdR was detected during storage (**Fig. IV-2**). Xt level was low but increased linearly from 0 nmol/g at time 0 to 12±4, 32±4, 60±6, 111±9, and 183±27 nmol/g after 1, 2, 4, 7, and 10 day of storage, respectively. The total content of ATP and its related compounds, 8.9±0.3 μmol/g, was constant during storage.

In the foot muscle of squid, ATP level at time 0, 2.1±0.2 μmol/g, was significantly lower than that in the mantle muscle (**Fig. IV-3**, $p<0.001$ with Student's t-test). ADP level decreased from the beginning of storage. A less extent of AMP accumulation was detected in the foot muscle compared with that in the mantle muscle. Hx level increased rapidly during storage. The total content of ATP and its related compounds in the arm muscle, 5.0±0.3 μmol/g, was significantly lower than that in the mantle

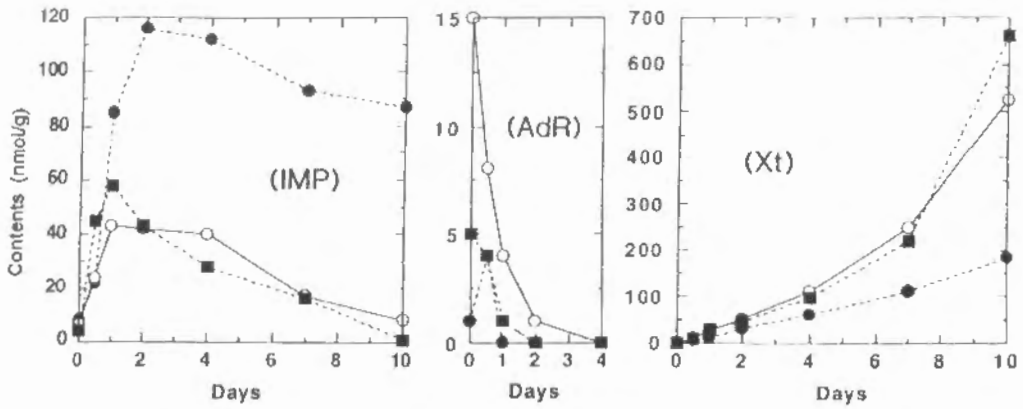

Fig. IV-2 Changes in average content of IMP, AdR, and Xt in the mantle (●), arm (■), and fin (○) muscles of spear squid during storage at 5° C (n=5).

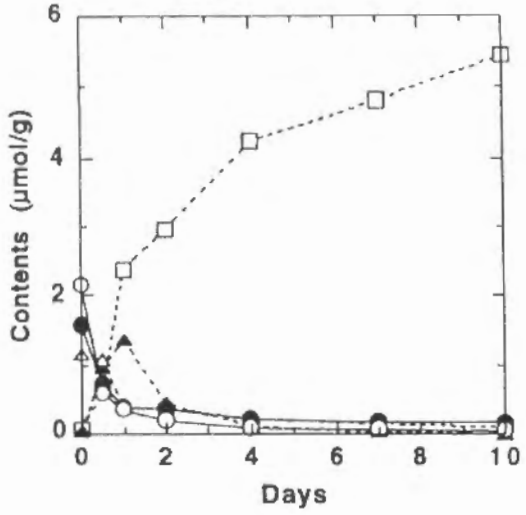

Fig. IV-3 Changes in average content of ATP and its related compounds in the arm muscle of spear squid during storage at 5° C (n=5). Symbols are the same as those given in the footnote of **Fig. IV-1**.

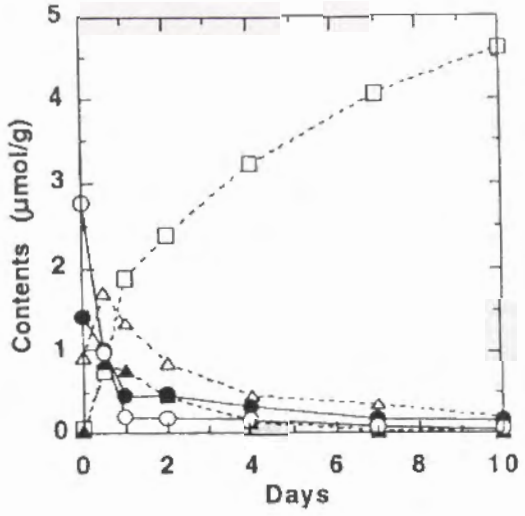

Fig. IV-4 Changes in average content of ATP and its related compounds in the fin muscle of spear squid during storage at 5° C (n=5). Symbols are the same as those given in the footnote of **Fig. IV-1**.

muscle ($p<0.001$ with Student's t-test). In the fin muscle, the patterns of changes in ATP and its related compounds were intermediate between those in the mantle muscle and arm muscle (**Fig. IV-4**). The total content of ATP and its related compounds was 5.2±0.3 μmol/g in the fin muscle. Small amounts of IMP and AdR were detected both in the arm and fin muscles (**Fig. IV-2**). Xt level increased faster during storage in those muscles than that in the mantle muscle. In the arm muscle, Xt level increased linearly from 0 nmol/g at time 0 to 248±34 nmol/g after 7 day of storage, and thereafter increased rapidly to 525±96 nmol/g after 10 day of storage. In the fin muscle, Xt level also increased linearly from 0 nmol/g at time 0 to 220±24 nmol/g after 7 day of storage, and thereafter increased rapidly to 661±145 nmol/g after 10 day of storage.

Chemical Freshness Indices

Figure IV-5 shows the changes in the K, K', and AEC values and Hx ratio with the sensory ratings on the mantle, arm, and fin muscles. On the 7th day of storage, the muscle gave a faintly putrid smell and this stage was recognized as the stage of initial decomposition. On the mantle, the K value increased and reached 79±2% at the stage of initial decomposition (**Fig. IV-5**). There was a significant difference among the values on the 0, 1st, 2nd, and 4th day of storage ($p<0.01$ with Student's t-test or $p<0.001$ with the paired t-test). There was also a significant difference between the values on the 4th and 7th day of storage with the paired t-test ($p<0.001$), but not with Student's t-test ($p>0.05$). The changes in the K' value on the mantle were similar to those in

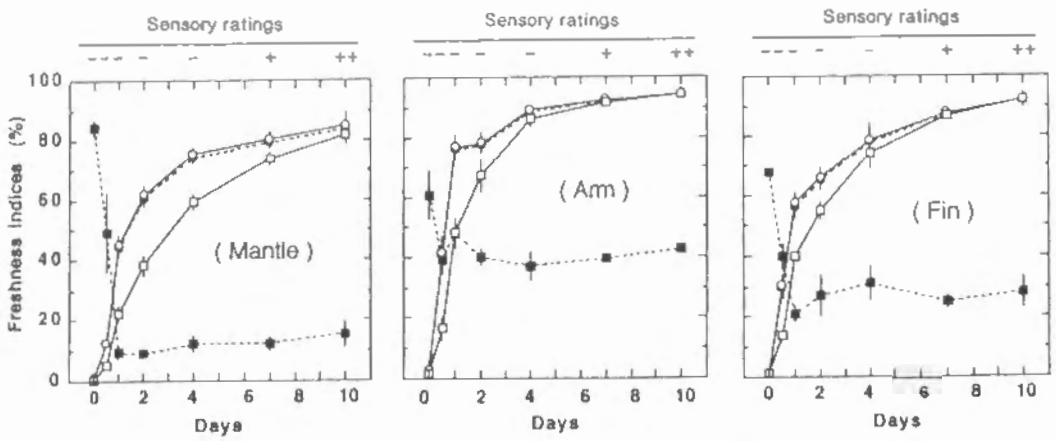

Fig. IV-5 Changes in average K (●), K' (○), and AEC (■) values and Hx ratio (□) in the mantle, arm, and fin muscles of spear squid during storage at 5° C (n=5) together with sensory ratings. -, acceptable; +, initial decomposition; and ++, advanced decomposition. Vertical bars indicate the standard deviation.

the K value. On the other hand, the Hx ratio on the mantle increased linearly compared with the K and K' values. There were significant differences among the ratios on the 0, 1st, 2nd, 4th, and 7th day of storage ($p<0.001$ with the paired t-test or $p<0.01$ with Student's t-test). The AEC value obtained on the mantle, fell from 84±1% to 9±2% by 1 day of storage, and then remained constant at low levels. The K and K' values obtained on the arm muscle increased rapidly from 3±1% to 40±4% within 4 h of storage, reached 75±2% on the 1st day, and thereafter increased slowly (**Fig. IV-5**). The Hx ratio increased linearly during 4 days of storage compared with the changes in the K and K' values. There were significant differences among the ratios on the 0, 1st, 2nd, and 4th day of storage ($p<0.01$, Student's t-test), while there was no difference between those on the 4th and 7th day of storage ($p>0.05$). The AEC value decreased rapidly from 60±5% to 40±2% during 4 h of storage. The changes of K, K', and AEC values or Hx

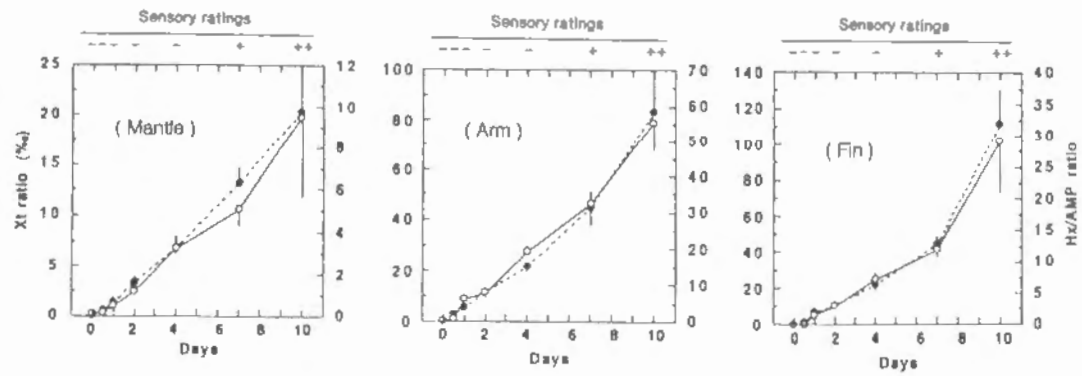

Fig. IV-6 Changes in average Xt (●) and Hx/AMP (○) ratios in the mantle, arm, and fin muscles of spear squid during storage at 5° C (n=5) together with sensory ratings. -, acceptable; +, initial decomposition; and ++, advanced decomposition. Vertical bars indicate the standard deviation.

ratio obtained on the fin muscle were intermediate between those obtained on the mantle and arm muscles (**Fig. IV-5**). There were significant differences among the K and K' values and Hx ratio on the 0, 1st, 2nd, 4th, and 7th day of storage ($p<0.05$, Student's t-test).

Figure IV-6 shows the changes in Xt and Hx/AMP ratios together with sensory ratings obtained on the mantle, arm, and fin muscles. Both ratios increased linearly and rapidly during storage of each tissue. Although the increasing rate was different among the tissues, the magnitudes of change in the two ratios were greater than those in the K and K' values or Hx ratio.

Discussion

The author previously showed that the total contents of ATP and its related compounds differed among the tissues of the oyster;

adductor muscle, mantle, gill, and body trunk in Section II-2. Watanabe et al. (1993) also reported that those contents were different among the disk abalone tissues; adductor muscle, foot muscle, mantle, and viscera. In this study, the total content of ATP and its related compounds in the mantle muscle was about 9 μmol/g, much higher than those in the arm and fin muscle, about 5 μmol/g. The average AEC value at time 0 obtained on the mantle muscle of spear squid was about 84% (**Fig. IV-5**), the same level as that reported by Saito et al. (1958) on the muscle of common squid Todarodes pacificus which was freshly killed and treated immediately. The value was much higher than those obtained on the muscle of the spear squid (Arai, 1966), "kensaki-ika" D. kensaki (Iwamoto and Uchiyama, 1969), and "jindo-ika" Loligo japonica (Suwetja et al., 1989) (ca. 56, 5, and 8%, respectively, which were calculated from the contents of ATP, ADP, and AMP in these reports). The energy charge implies that the conservation of ATP is a major feature of metabolic regulation Atkinson, 1968). The high level of AEC obtained on the mantle muscle in this study indicated that the specimens used in this study were in a good physiological condition before the experiment. On the other hand, the levels of ATP and AEC values at time 0 in the arm and fin muscles were lower than those in the mantle muscle (**Figs. IV-1, 3, 4, and 5**). One of the possible reasons for this phenomenon was that the arm and fin are sensitive to handling stress, such as netting.

The author described that the level of ATP in the muscle of unstressed carp decreased gradually from 7.3 μmol/g at time 0 to 6.9, 6.1, and 4.7 μmol/g after 1, 3, 8 h of storage at 25°C, respectively in Section I-1. In the mantle muscle of squid, the

ATP decreased much rapidly from 6.5 to 2.9 μmol/g within 4 h storage even at 5°C storage (**Fig. IV-1**), suggesting higher ATPase activity in the tissue. The levels of ADP and AMP in the muscle of spear squid were higher than those in the carp muscle, as reported in the muscle of common squid (Arai, 1966) and spear squid (Saito et al., 1958). The Hx increased rapidly in the muscles of mantle, arm, and fin from the beginning of storage as reported in the muscle of common squid (Arai, 1958; Nakamura et al., 19885). As for the pathway of AMP degradation, Arai (1958), Iwamoto and Uchiyama (1969), and Ohashi et al. (1991) analyzed the muscles of squid and reported that IMP did not accumulate during storage and that AMP degradation proceeded as AMP → AdR → HxR. On the other hand, Nakamura et al. (1985) and Suwetja et al. (1989) reported the presence of IMP in the muscle of squid. In this study, both IMP and AdR were detected in all muscles (**Fig. IV-2**). Obviously, AMP was degraded through the two pathways, IMP and AdR pathways, as shown in the muscles of other mollusca , such as oyster, ark-shell, and abalone (Chapter II and III).

As possible chemical freshness indices, the author calculated 6 indices, K, K' and AEC values, and Hx, Hx/AMP, and Xt ratios. The index for freshness is required to give information on the freshness before the onset of decomposition. The AEC value obtained on the muscles of mantle, arm, and fin could not be applied as a freshness index to spear squid, since it decreased rapidly within 1 day of storage and thereafter did not show any marked change (**Fig. IV-5**). Ohashi et al. (1991) and Nakamura et al. (1985) reported that the K value was not suitable as a freshness index on the muscle of common squid due to its rapid

increase after catch. In this study, the K and K' values on the arm muscle also seemed to be unsuitable as a freshness index (**Fig. IV-5**). However, K and K' values obtained on the mantle and fin muscles increased continuously during the acceptable stage (**Fig. IV-5**). From the changes of the values, the two values seemed to be useful as early freshness indices of spear squid. The Hx ratio on the mantle and fin muscles increased continuously throughout the storage period (**Fig. IV-5**) and seemed to be more suitable as a freshness index than K, K', or AEC value.

Ohashi et al. (1991) reported that the changes in the Hx/AMP ratio are higher than those in K value and suggested that this ratio is more useful as a freshness index than the K value in common squid. In this study, the change in Hx/AMP ratio was linear with the storage time and the magnitude of the change was larger than that in K or K' values in the three muscles of spear squid (**Fig. IV-6**). The change in Xt ratio was linear with the storage time and the magnitude of the change was also as high as that of the Hx/AMP ratio. Although micro analysis was needed because of the small content of Xt in the muscles (**Fig. IV-2**), the Xt ratio seemed to be useful as a freshness index for spear squid. In conclusion, the K and K' values could be used to assess freshness of spear squid especially in earlier period of storage, while the Hx, Hx/AMP, and Xt ratios were better indices of the freshness of spear squid throughout the storage period.

Chapter V. Summary and Conclusions

The author investigated the postmortem changes in high-energy phosphate compounds and discussed chemical indices for assessing freshness of fish and shellfish. The effects of anesthetic stress on the postmortem levels of ATP and its related compounds was tested in carp muscle by HPLC. The decrease of ATP from 7.3μmol/g to 4.5 and increase of IMP from 0.3μmol/g to 1.8μmol/g occurred in the unstressed and stressed muscle, respectively (Section 1 of Chapter I). The anesthetic stress accelerated the postmortem changes of ATP and its related compounds of carp muscle. Using ^{31}P NMR, the author also examined the postmortem changes of high-energy phosphates in unstressed and stress fish (Section 2 of Chapter I). The PCr acts as a energy reservoir even in the muscle after death; the reasons for the differences in ATP levels between the stressed and unstressed carp muscle are the decrease of PCR level, energy reservoir of ATP, of the stressed carp and the differentiated ability of glucose utilization because of the substantial hypoxic stress. Additionally, judging from the several parameters obtained simultaneously by ^{31}P NMR, such as the ratios of high energy phosphates to Pi and pHi, it was possible to evaluate meat quality such as freshness of carp muscle and also the energy state of the muscle including the fatigue of the fish i.e., whether the fish was stressed or unstressed before death. These results suggested that the ^{31}P NMR is a possible tool for the evaluation of fish freshness, because of its non-invasive, convenient, rapid, and simultaneous determination of high energy phosphate compounds, Pi, and pHi in the tissue of fish.

The author showed that the pathway, patterns, and rates of postmortem changes and the contents of ATP and its related compounds of mollusk were different from those of fish muscle and among species and tissues (Chapter II, III, and IV). ATP decreased gradually in the mantle, gill and body trunk of oyster, ark-shell muscle, and abalone muscle, while it decreased rapidly in the muscle of oyster, hard clam, and spear squid. AMP was found to accumulated, in each tissue or species. Further degradation occurred obviously in the foot muscle of ark-shell to IMP, in the adductor muscle of oyster to HxR, and in the mantle, fin and arm muscles of spear squid to Hx. These findings suggested that the activities of the enzyme systems responsible for the degradation of ATP, ADP, AMP, and IMP, that is, ATPase, myokinase, AMP deaminase, and 5'-nucleotidase, respectively, differed among the species and also tissues. Both IMP and AdR were detected in the muscle of the oyster, ark-shell, abalone, and spear-squid. The author found two pathways of AMP degradation, IMP pathway and AdR pathway, in those kinds of mollusks during storage. The author also found the interesting effects of storage temperature on postmortem changes of ATP in oyster muscle, i.e. a faster degradation of ATP at lower storage temperature.

The freshness index of fish and shellfish should change linearly and continuously with certain magnitudes during acceptable stage. The K value, a well-known chemical index of fish freshness, was considered to be unsuitable because of the very slow increase at the acceptable stage in pelecypod (Chapter II) and gastropod (Chapter III). In the case of oyster, although the tissues latently have the strong endogenous activities of ATP-breakdown

to HxR and/or Hx, when the tissue structure is maintained, the enzymatic activities of ATP-breakdown are limited from ATP to AMP and/or IMP and further breakdown proceed very slowly. From these properties in endogenous enzymes of ATP-breakdown, the K value showed the slow increase and was considered unsuitable for the freshness index for oyster (Section 4 of Chapter II). The K value was also unsuitable due to the rapid increase immediately after death in cephalopod (Chapter IV). The K' and/or AEC values were shown to be potential indices for pelecypod and gastropod, and also Hx, Xt, and Hx/AMP ratios for cephalopod. The author also showed that the ^{31}P NMR is a possible tool to evaluate the oyster freshness, because of its non-invasive, convenient, rapid, and simultaneous determination of high energy phosphate compounds, Pi, and pHi in the tissue of oyster.

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